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(54) Title: **METHODS FOR RAPID PEG-MODIFICATION OF VIRAL VECTORS, COMPOSITIONS FOR ENHANCED GENE TRANSDUCTION, COMPOSITIONS WITH ENHANCED PHYSICAL STABILITY, AND USES THEREFOR**

(57) Abstract: A rapid method for modifying a viral capsid or envelope protein with a polyethylene glycol (PEG) is described. Also provided are methods of delivering a molecule using the PEG-modified viruses of the invention. Compositions containing the PEG-modified viruses of the invention, are characterized by improved gene expression, reduced neutralizing antibody and CTL production. Also provided are viral compositions having enhanced physical stability, in which the viruses are lyophilized in a formulation having a 1:1 ratio of sucrose and mannitol are provided.

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METHODS FOR RAPID PEG-MODIFICATION OF VIRAL VECTORS,
COMPOSITIONS FOR ENHANCED GENE TRANSDUCTION,
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AND USES THEREFOR

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government may have certain rights in this invention.

Field of the Invention

10 The invention relates generally to the field of gene delivery into host cells,
and more particularly, to gene delivery via viral vectors.

Background of the Invention

15 Viral-mediated gene delivery has been described for delivery of therapeutic
genes to patients. One limitation of currently known methods is the generation of
neutralizing antibodies (NAB) by the patients immune response against viral capsids,
which prohibit significant levels of gene expression upon readministration. Thus,
methods of gene delivery which circumvent these immune responses are needed.

20 Covalent modification of proteins and enzymes with functionalized
poly(ethylene) glycol (PEG) has been studied. PEG is an uncharged, hydrophilic,
linear polymer that is non-immunogenic and has a very low order of toxicity.
Recently, O'Riordan et al have developed a process to covalently link various
polyethylene glycols to the capsid proteins of adenovirus [O'Riordan et al, Hu Gene
Therapy, 10:1349-1358 (1999)]. However, this method requires incubation for a
period of 20 to 24 hours.

25 What is needed are viral vectors which avoid the limitations of current
constructs for re-administration, as well as rapid methods for generating high levels
of such constructs.

Summary of the Invention

In one aspect, the invention provides a method for conjugating a recombinant virus with polyethylene glycol to enhance transduction efficiency thereof. This method involves the steps of reacting activated PEG and a recombinant virus at room temperature for about 15 minutes to about 2 hours; and stopping the reaction, thereby obtaining PEG-conjugated virus. Most suitably, the activated PEG and the recombinant virus are reacted at a ratio of about 10: 1 polyethylene glycol to virus. Desirably, the recombinant virus is present at a concentration of about 1×10^{10} to about 1×10^{15} particles per ml of reaction solution.

10 In another aspect, the invention provides a PEG-conjugated virus prepared according to the method of the invention.

In yet another aspect, the invention provides a method for increasing transduction efficiency of a recombinant virus which involves delivering a modified recombinant virus according to the invention to host cells.

15 In still another aspect, the invention provides a method for re-administration of a molecule to a selected host cell via a viral vector. This method involves the steps of contacting the host cell with a PEG-modified virus according to the invention, wherein said virus comprises a molecule for delivery to a host cell; and contacting the host cells with a recombinant virus comprising the molecule.

20 In yet a further aspect, the invention provides a composition useful for delivery of a selected molecule to host cells. The composition contains a PEG-conjugated virus prepared according to the method of the invention and a physiologically acceptable carrier.

In still a further aspect, the invention provides a composition that enhances the physical stability of viral vectors. This composition contains a recombinant viral vector comprising a molecule for delivery to host cells, sucrose, and mannitol, wherein the ratio of sucrose to mannitol is about 1 to about 1. Desirably, the composition is lyophilized to a final moisture content of about 1.2% to about 1.7%.

25 Other aspects and advantages of the invention will be apparent from the following detailed description of the invention.

30

Brief Description of the Drawings

Fig. 1A illustrates the results of a study on the effect of the final frozen pH of a formulation on adenovirus stability (correlation coefficient, $r^2 = 0.98$). See Example 15. The results are reported as the average titer of 4-6 samples from two separate experiments. Error bars reflect the standard deviation of the data.

Fig. 1B illustrates the results of a study on the effect of the final frozen pH of a formulation on adeno-associated virus stability (correlation coefficient, $r^2 = 0.98$). See Example 15. The results are reported as the average titer of 4-6 samples from two separate experiments. Error bars reflect the standard deviation of the data.

Fig. 1C illustrates the results of a study of the stability of adenovirus prepared in either sodium (DPBS) or potassium (KPBS) phosphate buffered saline and 10% glycerol at -20°C .

Fig. 2 illustrates the results of a study of the effect of the addition of excipients on adenoviral preparation on the recovery of active vector after lyophilization. Pre-lyophilization (Pre-lyo) titer for the entire lot was 1×10^{11} lfu/ml. Concentrations of sucrose, mannitol and Span 20 in the Suc/Man/Span formulations are 40 mg/mL, 40 mg/mL and 0.001%, respectively. K = KPBS, D = DPBS. Data are the average of eight vials from two separate experiments. Error bars represent the standard error of the data.

Detailed Description of the Invention

The invention provides a novel, rapid, method of modifying a protein or enzyme with an activated polyethylene glycol (PEG) polymer. Advantageously, the modification method described herein is complete within a short period of time (e.g., about 1 to 2 hours), occurs under mild conditions (e.g., room temperature, physiologic pH), and enhances physical stability under extreme storage conditions.

This method has been found to be particularly well suited for modification of the capsid protein of viral vectors. Advantageously, substantial gene expression has been obtained using the PEG-modified viral vectors of the invention upon re-administration intravenously and intramuscularly without compromising the recipient's immune system against other pathogens. The vectors of the invention are

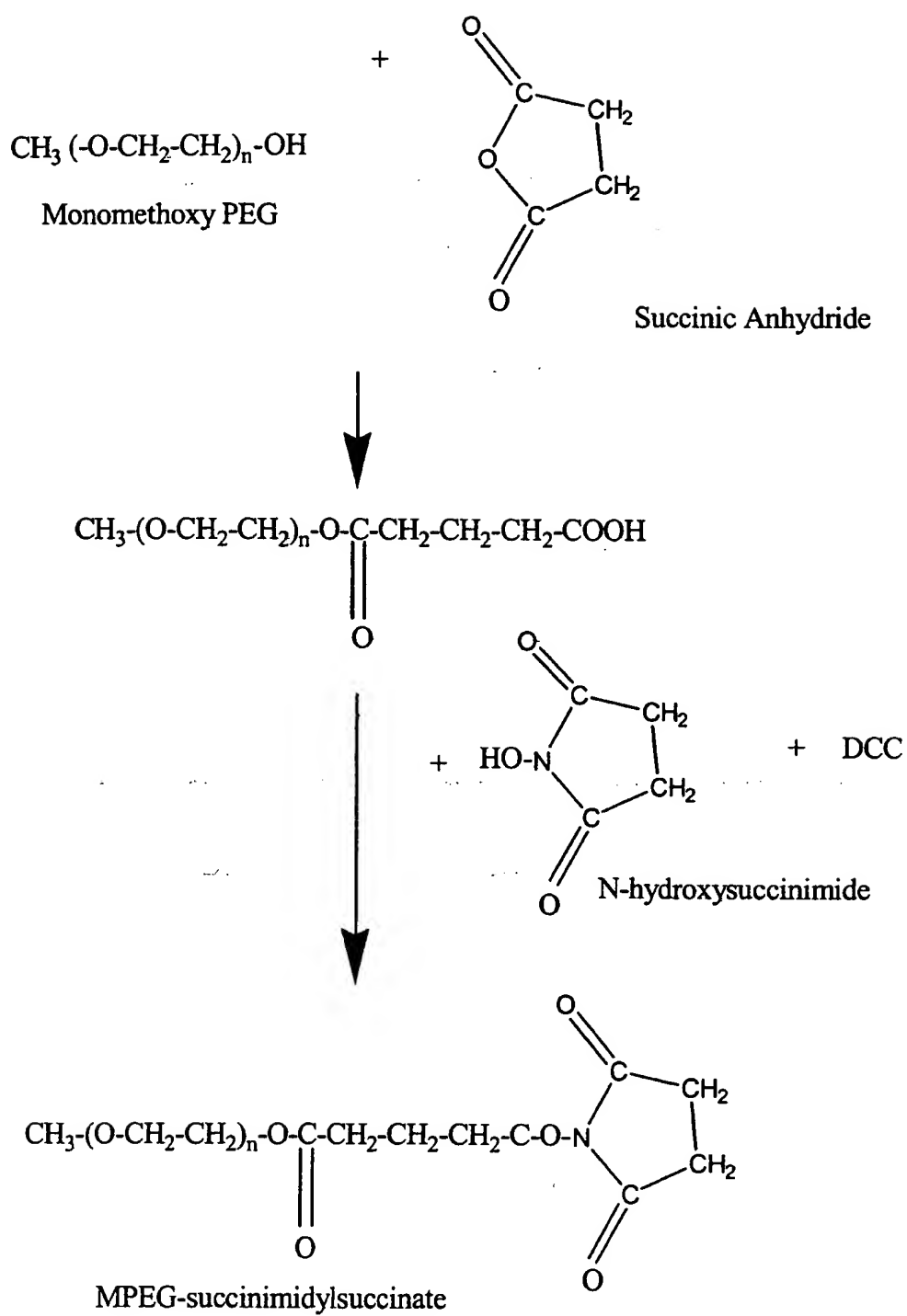
also useful for delivery by other routes. The modified vectors of the invention are particularly useful in therapies that require chronic treatment. Further, these modified vectors allow for significant levels of gene expression in patients with high titers of neutralizing antibodies. In addition, re-administration can occur with the native virus after prior exposure to the modified virus.

Thus, the PEG-modification method of the invention is particularly well suited for modification of recombinant viruses which contain a desired molecule for delivery to a host cell within a capsid or envelope protein. In one particularly desirable embodiment, the molecule for delivery is a nucleic acid sequence encoding a transgene under the control of regulatory sequences which direct its expression in the host cell. In another desirable embodiment, the molecule for delivery is a protein, chemical, enzyme, or other moiety. Selection of the molecule for delivery is not a limitation of the present invention.

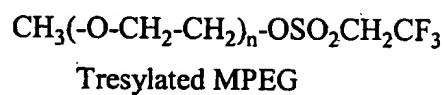
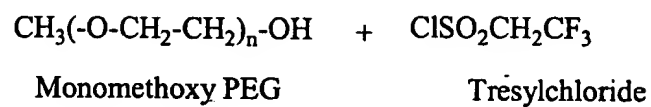
In a particularly desirable embodiment, the methods of the invention are used in connection with adeno-associated viruses. However, these methods may be readily applied to other viruses which contain their genetic material within capsid or envelope proteins. Examples of other suitable viruses having capsids or envelopes suitable for PEG-modification include, adenoviruses, retroviruses, lentiviruses, among others. For convenience throughout the specification, reference will be made to capsid proteins. However, it will be understood that similar methods may be applied to modify viral envelope proteins. Similarly, the methods of the invention may be readily used to modify other proteins and enzymes, whether viral or non-viral. As defined herein, polyethylene glycol (PEG) is a polymer composed of repeating units of the formula: $\text{H}-(\text{OCH}_2\text{CH}_2)_n-\text{OH}$, where n is 2 to about 1000. Most suitably, the invention uses a PEG compound having a molecular weight in the range selected from about 500 to about 500,000, about 1000 to about 200,000, and about 5000 to 100,000. Encompassed within this definition are substituted PEG compounds. In a currently preferred embodiment, the substituted PEG is monomethoxypolyethylene glycol (MPEG) having a molecular weight of 5000. MPEG has the formula $\text{CH}_3-(\text{OCH}_2\text{CH}_2)_n-\text{OH}$ and may be generated by conventional synthesis techniques or purchased commercially. Other suitable substituted PEG

compounds can be readily determined, or designed, particularly to present a chemical group suited to activation and/or as a point of attachment to the viral protein capsid.

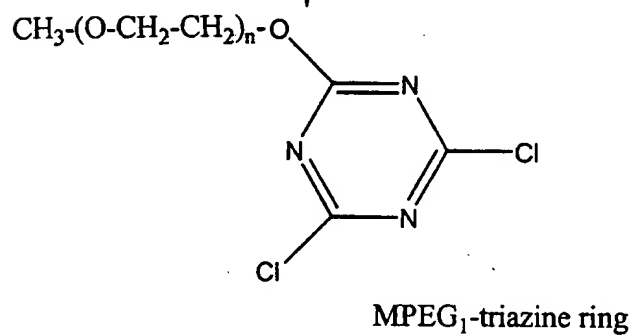
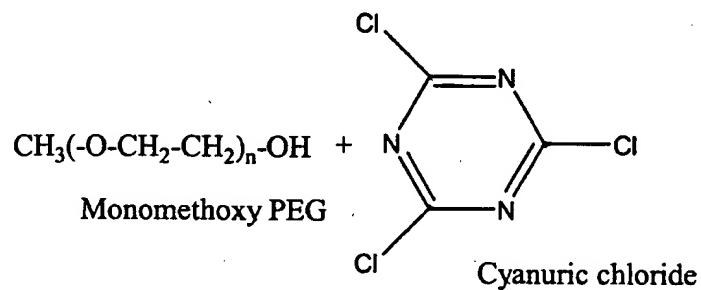
- 5 MPEG (or another selected PEG) is chemically activated prior to coupling to the selected protein (e.g. virus capsid) or enzyme. The chemical activation may be performed using conventional techniques. Activated MPEG may be purchased from a variety of commercial sources. For example, tresyl-MPEG (TMPEG), succinimidyl succinate-MPEG (SSMPEG), and cyanuric chloride-MPEG (CCMPEG) may be obtained from Sigma Chemicals (St. Louis, MO). Alternatively,
- 10 MPEG may be activated with succinimidyl activate esters according to the following scheme:



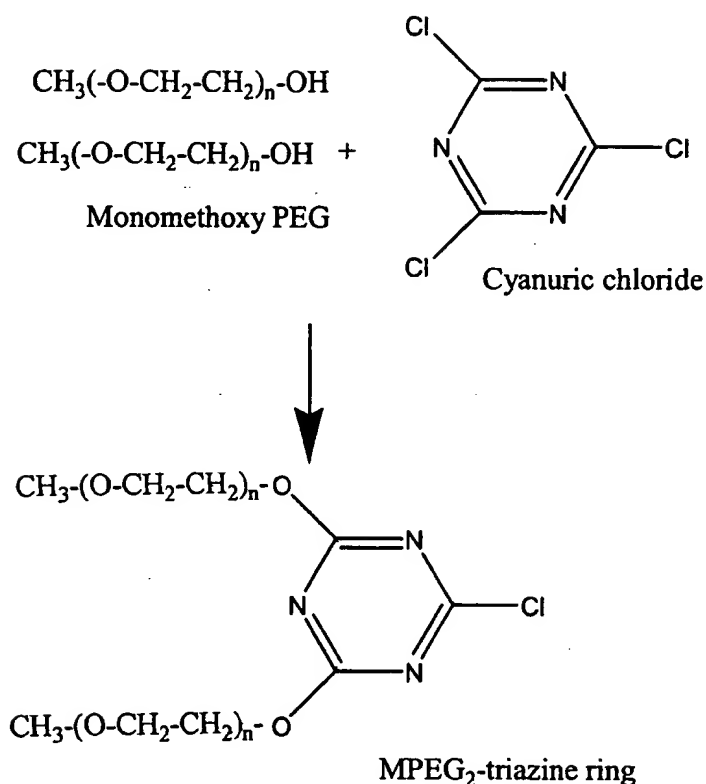
MPEG may be activated with tresyl chloride according to the following scheme:



MPEG may be activated with cyanuric chloride according to one of the following two schemes:



Another suitable scheme for activation of MPEG with cyanuric chloride is as follows:



- Regardless of the activation compound or method utilized, the activated
- 5 MPEG is thereafter conjugated with the selected protein (e.g., viral capsid) using the method of the invention, which is more rapid and performed under less stringent conditions, as compared to the prior art methods [see, e.g., Delgado, *Biotechnol. Appl. Biochem.*, 12:119-128 (1999)]. More particularly, the methods in the art
- 10 required incubation for a period of 20 to 24 hours, and in some cases involved the step-wise addition of varying concentrations of activated PEG at regular intervals during the incubation period. The resulting product was a "super-pegylated virion". Advantageously the method of the invention eliminates this requirement for addition

of activated PEG at regular intervals, and provides a PEG-modified product in about 1 to 2 hours.

According to the method of the invention, the protein selected for modification (e.g., the viral capsid) is preferably purified from any contaminants.

5 For example, a viral vector may be produced using methods known to those of skill in the art [See, e.g., K.J. Fisher et al, *J. Virol.*, 70:520-532 (1996)]. Thereafter, the viral vector is preferably purified by conventional methods. For example, cesium chloride (CsCl) gradients [K. J. Fisher et al, *Nat. Med.*, 3:306-312 (1997)] and column chromatography methods are particularly desirable. A variety of columns
10 useful for purification are available commercially (e.g., Bio-Rad, Perseptive Biosystems, etc.) and other methods have been described in the literature. Selection of the method of purification is not a limitation of the present invention.

Suitably, the construct (e.g., purified viral vector) selected for PEG-modification is desalted to remove any residual salt from the purification process
15 (e.g., CsCl) or storage medium. In one desirable embodiment, this desalting is performed on a commercially available chromatography column. Following desalting, the viral vector is equilibrated with a buffer compatible with the PEG-conjugation reaction described below, or a formulation that enhanced viral stability upon long-term storage. Suitably, the vector may be equilibrated with the buffer
20 selected for use in the conjugation reaction. The buffer may be selected taking into consideration such factors as convenience and the type of activated PEG used in the reaction.

For example, when the construct is to be conjugated with TMPEG according to the invention, a particularly desirable buffer is potassium phosphate buffer
25 (KPBS) at a pH of about 7 to about 8, and most preferably 7.4. For conjugation with SSMPEG and CCMPEG, purified protein (e.g., bands from the CsCl gradients) are desalted in sodium phosphate (pH about 6.5 to about 7.5, and most preferably 7.2) and sodium tetraborate (pH about 8.5 to about 9.9, and most preferably 9.2) buffers. However, given these parameters, one of skill in the art may adjust these buffers as
30 needed.

Once the protein is desalted (if necessary) and equilibrated with the selected buffer, the selected activated PEG is combined with the virus to form a reaction mixture of PEG and protein, in which the activated PEG is present in a PEG-to-virus ratio in the range of 1:1 to 20:1, and about 5:1 to 15:1. Where the virus is rAAV, a ratio of 10:1 is preferred. However, one of skill in the art can readily adjust these ratios, as desired. Suitably, the reaction mixture is performed at room temperature (e.g., about 22°C to 24°C) under gentle stirring for about 15 minutes to about 120 minutes and the reaction is stopped. One desirable method of stopping the reaction are by adding an excess of L-lysine with respect to the amount of PEG. About a 10-fold excess has been found desirable; however, one of skill in the art could readily select another suitable amount. Alternatively, the reaction may be stopped by lowering the temperature of the reaction to about 4°C. Desirably, the reaction is allowed to proceed until complete modification (i.e., about 100%) modification of the target protein (e.g., viral capsid) is achieved. However, under certain circumstances, the reaction may be stopped when the modification is less than complete. For example, when certain activated CCPEGs are utilized, it may be desirable to stop the reaction when modification is about 70% complete, in order to avoid agglomeration which is observed with certain types of activated CCPEGs in certain proteins. However, as the advantages of the invention, which include increased transduction efficiency, have been observed with less than 100% modification, it may be desirable to stop the reaction at 70% to 98%, 75% to 95%, 80% to 90%, or 85% completion for a variety of reasons which will be readily apparent to one of skill in the art. The degree of modification can be determined using conventional methods. See, e.g., the fluorescamine assay described in Example 3 herein.

The resulting PEG-modified protein is separated from unreacted PEG, excess lysine and reaction byproducts. Examples of suitable separation methods include CsCl centrifugation or passage of the reaction mixture through a column equilibrated with a suitable buffer (e.g., 10 mM potassium buffered saline (KPBS) at pH 7.4). However, other separation methods may be readily selected. Fractions that contain

the PEG-modified construct may thereafter be confirmed by UV spectrophotometric analysis at 260 nm or other suitable methods. After separation (purification) the PEG-modified construct (e.g., virus) may be suspended in a solution suitable for storage and/or delivery to host cells.

5. Advantageously, the inventors have found that viruses modified by this method retain infectivity. Most suitably, the PEG-modified viruses of the invention avoid incorporation of the activating group into the modified virus, thus avoiding any problems associated with potential immunogenicity of the coupling moiety. Thus, the present invention further provides compositions containing PEG-modified
- 10 viruses of the invention and a carrier suitable for delivery of the composition to host cells for a variety of therapeutic and other purposes.

Compositions of the Invention

A. PEG-modified Constructs

- In one embodiment, a composition of the invention contains a PEG-
- 15 modified protein or enzyme of the invention and a physiologically compatible carrier. In a particularly desirable embodiment, the invention provides a composition containing a PEG-modified virus. Suitably such a virus encodes a transgene which is under the control of regulatory sequences which direct its expression in a host cell. Alternatively, the virus may carry another molecule for
- 20 which delivery to the host cell is desirable.

- Suitable doses of PEG-modified viruses may be readily determined by one of skill in the art, depending upon the condition being treated, the health, age and weight of the veterinary or human patient, and other related factors. However, generally, a suitable dose may be in the range of 10^{10} to 10^{18} , and preferably about
- 25 10^{14} to 10^{16} viral particles per dose, for an adult human having weight of about 80 kg. This dose may be suspended in about 0.01 mL to about 1 mL of a physiologically compatible carrier and delivered by any suitable means. The dose may be repeated, as needed or desired, daily, weekly, monthly, or at other selected intervals using any suitable delivery means.

Suitably, such a carrier is physiologically compatible, e.g. saline, distilled water, phosphate buffered saline (PBS), potassium (K) PBS, sodium PBS, and the like, suitable for administration to a human or non-human mammalian patient. Other exemplary carriers include sterile saline, lactose, sucrose, calcium
5 phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

Optionally, the compositions of the invention may contain, in addition to the PEG-modified virus and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, chemical stabilizers, or surfactants, may be
10 included in the formulation. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. For example, suitable chemical stabilizers may include gelatin and albumin. Optionally, a composition of the invention may contain free amino acids, e.g. adenosine, other
15 components such as carbohydrates, surfactants and other stabilizers.

B. Formulations which Enhance Physical Stability

In another aspect, the present invention provides novel formulations which enhances the physical stability of viral vectors even under harsh storage conditions. In one embodiment, these viral vectors are modified according to the
20 method of the present invention. However, the formulations described herein are useful for a variety of other viral vectors, which may be readily selected by one of skill in the art. In addition, the formulations described herein may be desirable for a variety of non-viral vectors, e.g., plasmids, or other DNA or proteins which will be subject to lyophilization, long-term storage under freezer or refrigeration conditions,
25 and shipping.

Suitably, for long-term storage, the viral vector is lyophilized to contain a final moisture content in the range of about 1.2% to 1.7%, and more preferably about 1.3% to 1.4%, in a formulation. Desirably, the formulations of the invention contain at least about 5×10^{10} to about 1×10^{16} , or about 5×10^{11} viral
30 particles/ml. Where non-viral constructs are utilized, one of skill in the art can

readily determine the appropriate concentration of the selected construct. For example, a formulation of the invention may contain about 0.1 μ g to about 10 mg DNA, more preferably about 10 μ g to about 1 mg DNA, or about 0.1 μ g to about 10 mg protein, more preferably about 10 μ g to about 1 mg protein. However, other
5 suitable concentrations may be readily selected by one of skill in the art. Using these parameters, lyophilization may be performed using conventional techniques. See, generally, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

In one particularly desirable embodiment, the construct to be
10 formulated into a composition of the invention (e.g., a viral vector) is combined in solution with sucrose and mannitol. For viral vectors, a ratio of about 1:1 sucrose to mannitol is particularly desirable. However, the ratio of sucrose to mannitol may be adjusted, and more desirably about 3:4 sucrose to mannitol, taking into consideration the stability of the selected construct on storage. For example, in general, an adeno-
15 associated viral vector tends to exhibit better stability than an adenoviral vector under long-term storage conditions. For example, ratios may range from about 4:1 sucrose to mannitol to about 1:4 sucrose to mannitol, and more desirably about 3:4 sucrose to mannitol, and most desirably, about 1:1.

Most preferably, a composition of the invention having enhanced
20 storage stability also contains about 0.5% to 5% beta cyclodextrin (BCD), and most preferably a tertiary amine BCD. In one particularly desirable embodiment, this composition further contains about 0.5 mg/ml to about 1 mg/ml pluronic protamine.

Optionally, the compositions of the invention may contain
conventional pharmaceutical ingredients, such as preservatives, carbohydrates,
25 stabilizers, or surfactants, such as those described above.

The compositions of the invention, including the PEG-modified
constructs of the invention whether formulated as described above and/or the
compositions formulated for long-term storage whether prepared with the PEG-
modified constructs or other constructs, may be delivered using methods known to
30 those of skill in the art. Where the PEG-modified vectors (or other constructs) have

been lyophilized, the vectors can be readily reconstituted using methods known to those of skill in the art. See, e.g., L. Rey and J. C. May, "Freeze-drying/lyophilization of pharmaceutical and biological products", Drugs and the Pharmaceutical Sciences, Vol. 96, 1999, Marcel Dekker: New York, New York;

5 M.J. Pikal, "Freeze-drying of Proteins", Part 1: Process Design., Biopharm, 1990, September: p. 18-27; M.J. Pikal, Biopharm., 3(8):28-31 (1990).

Methods of the Invention

The invention provides a method of delivering a transgene or other molecule

10 to a human or veterinary patient by delivering a PEG-modified construct to the patient. The target cells may be transduced *in vivo* or *ex vivo*, taking into consideration such factors as the selection of target cells and the condition for which the patient is being treated. For convenience, the following discussion will refer to delivery of transgenes by PEG-modified viral vectors. However, these methods may

15 also be used to deliver other PEG-modified constructions and the other compositions of the invention.

A. In vivo

For *in vivo* delivery of the transgenes, any suitable route of administration may be used, including, direct delivery to the target organ, tissue or

20 site, intranasal, intravenous, intramuscular, subcutaneous, intradermal, vaginal, rectal, and oral administration. Routes of administration may be combined within the course of repeated therapy or immunization.

Suitable doses of PEG-modified viruses may be readily determined by one of skill in the art, depending upon the condition being treated, the health, age

25 and weight of the veterinary or human patient, and other related factors. However, generally, a suitable dose may be in the range of 10^3 to 10^{18} , preferably about 10^5 to 10^{16} particles per dose, for an adult having a weight of about 80 kg. This dose may be formulated in a pharmaceutical composition, as described above (e.g., suspended in about 0.01 mL to about 1 mL of a physiologically compatible carrier) and

delivered by any suitable means. The dose may be repeated, as needed or desired, daily, weekly, monthly, or at other selected intervals.

B. Ex Vivo

In another embodiment, the PEG-modified viruses of the invention are useful for *ex vivo* transduction of target cells. Generally, *ex vivo* therapy involves removal of a population of cells containing the target cells, transduction of the cells *in vitro*, and then reinfusion of the transduced cells into the human or veterinary patient. Such *ex vivo* transduction is particularly desirable when the target cells are dendritic cells or macrophages and/or when the transgene or other molecule being delivered is highly toxic, e.g., in the case of some genes used in the treatment of cancer. However, one of skill in the art can readily select *ex vivo* therapy according to the invention, taking into consideration such factors as the type of target cells to be delivered, the molecule to be delivered, the condition being treated, the condition of the patient, and the like.

Generally, when used for *ex vivo* therapy, the targeted host cells are infected with 10^5 viral particles to 10^{10} viral particles for each 10^1 to 10^{10} cells in a population of target cells. However, other suitable *ex vivo* dosing levels may be readily selected by one of skill in the art.

C. Re-Administration

In one embodiment, the invention provides methods of delivering transgenes via PEG-modified viral vectors, in which re-administration or repeat delivery is performed using a PEG-modified vector of the invention in which the activating group of the MPEG differs from the activating group utilized on the PEG-modified vector of the first administration. Thus, the invention may involve delivering a PEG-modified viral vector conjugated with first activated MPEG (e.g., tresyl-MPEG) and a second delivery with the PEG-modified viral vector in which the MPEG is conjugated with a second activated MPEG group (e.g., succinimidyl succinate MPEG, SSPEG). Alternatively, a PEG-modified viral vector of the invention may be used in a dosage regimen in which non-pegylated viral vectors are utilized. Thus, a PEG-modified viral vector of the invention may be delivered prior

to delivery of a non-pegylated viral vector. In another example, a delivery of a PEG-modified viral vector of the invention may follow a prior administration with a non-pegylated viral vector.

5 The following examples are provided to illustrate the methods of preparing the compositions of the invention and the methods of the invention. These examples do not limit the scope of the invention. One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following examples, modifications can be made which are meant to be encompassed by the spirit and
10 scope of the invention.

Example 1 - Production of Conjugated Adeno-Associated Virus (AAV) Vectors

A. Preparation of Recombinant AAV (rAAV)

 rAAV containing LacZ were prepared according to published
15 methods. An rAAV used in the following studies, AAVCMVLacZ, contains AAV 5' and 3' ITRs flanking an *E. coli* β -galactosidase reporter gene expressed under the control of a human cytomegalovirus (CMV) promoter. Another rAAV used in these studies is AAV α 1AT, which contains AAV 5' and 3' ITRs flanking a human α 1-anti-trypsin (α 1AT) gene expressed under the control of the albumin promoter.
20 These were produced using the B50 cell line as described [G.-P. Gao et al, Hum. Gene Ther., 9:2353-2362 (1998)].

 B50 cells (a HeLa based cell line expressing AAV Rep/Cap proteins) were infected with wild-type Ad5 at a multiplicity of infection of 10 for 24 hours and then with an Ad-AAV hybrid vector [K. J. Fisher et al, J. Virol, 70:520-532 (1996)] at the
25 same multiplicity of infection (MOI) for an additional 48 hours. At this time, cells were harvested, resuspended in 10 mM Tris buffer pH 8.1 and lysed by three freeze/thaw cycles in dry ice/ethanol and 37°C baths. Benzonase (Nucomed Pharma, pure grade) was added to the mixture (50 U/ml, final concentration) and the lysate incubated at 37°C for 30 minutes. Lysate was clarified by centrifugation at

3700g for 20 minutes and supernatant collected. According to established methods [S. Zolotukin et al, Gene Ther., 6:973-985 (1999)], lysate was loaded on a POROS HE 20 (Perseptive Biosystems) column equilibrated with 20 mM PBS (pH 7.5), 250 mM NaCl. After the lysate entered the resin, the column was washed with 10 column volumes of the equilibration buffer. Vector was eluted from the column directly onto a POROS 50 PI column with 20 mM PBS (pH 5.5) 400 mM NaCl. Fractions from the second column were collected, concentrated, and incubated at 56°C for 10 minutes to inactivate any residual adenovirus in the preparation.

B. Conjugation of Viral Vectors

A monomethoxy derivative of polyethylene glycol (approx. MW 5000) was chosen for conjugation to the rAAV particles. This compound must first be chemically activated prior to the conjugation reaction. Three types of activated monomethoxypolyethyleneglycol (MPEG) were used in this study: tresyl-MPEG (TMPEG), succinimidyl succinate MPEG (SS-PEG), and cyanuric chloride MPEG (CC-MPEG). All activated PEGs were obtained from Sigma Chemicals (St. Louis, MO). In all cases, conjugation reactions were performed using a modification of established methods [Delgado, Biotechnol. Appl. Biochem., 12:119-128 (1990); Kita, Drug Design & Delivery, 6:157-167 (1990); Jackson, Analytical Biochem., 165:114-127 (1987)]. For conjugation with TMPEG, column purified AAV was dialyzed in 10 mM potassium phosphate buffer pH 7.4. Viral bands were desalted into 0.2 M sodium phosphate (pH 7.2) and 0.1 M sodium tetraborate (pH 9.2) buffers for conjugation reactions with SS-PEG and CC-PEG respectively. The inventors found that for conjugation by each type of PEG, a 10:1 (300 µg/3 mg) PEG:virus ratio (amt PEG:amt AAV protein) provided highly efficient reaction times and produced minimal loss of infectivity of the virus. AAV mixed with unactivated MPEG, a polymer that cannot covalently attach to the virus, served as a control for all *in vitro* and *in vivo* transduction experiments. All conjugation reactions were performed at room temperature under gentle stirring. Reactions were stopped by addition of a 10-fold excess (with respect to PEG concentration) L-lysine. Unreacted PEG, excess lysine, and reaction byproducts were eliminated by passing

the preparation through a Sephadex G-50 column equilibrated with 10 mM potassium buffered saline (KPBS) pH 7.4. Fractions containing virus were identified by UV spectrophotometric analysis at 260 nm and pooled for further study.

Example 2 - Infectivity Assay for Conjugated and Unconjugated Virus

5 Aliquots of conjugated and unconjugated AAV were serially diluted in DMEM supplemented with 2% FBS and added to 293 cells. Two hours after infection, preparations were removed and replaced with complete medium. Twenty hours after infection, cells were washed with PBS, fixed with 0.5% glutaraldehyde, and washed twice with PBS containing 1 mM $MgCl_2$. β -galactosidase expression
10 was determined by incubation with 1 mg/ml of the substrate, 5-bromo-4-chloro-3-indolyl- β -galactoside (X-gal) in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, $3H_2O$, and 1 mM $MgCl_2$ for 2 hours at 37°C in the dark. Lac^+ cells were coated from a minimum of 20 microscope fields. Percent infectivity was determined by calculating the ratio of the number of Lac^+ cells at various timepoints to the number
15 of Lac^+ cells at the initiation of the study.

Unconjugated AAV experienced the most significant loss in titer (29%) over the entire reaction period. The CCPEG vector was the least stable of all the PEGylated preparations with losses similar to that of the native virus. Simple addition of PEG to a preparation does not compromise viral titer as addition of
20 unactivated MPEG to the viral preparation did not significantly affect infectivity. Upon further investigation, it was found that the loss of infectivity of the CCPEG vector can be attributed to the formation of large aggregates due to crosslinking of multiple virions. However, reacting the vector with CCPEG for a shorter period of time yields fewer aggregates and even though less than 100% modification is
25 achieved, transduction efficiency and stability are greatly enhanced.

Example 3 - Fluorescamine Assay

A fluorescamine assay was used to estimate the degree of modification of AAV capsids by activated polyethylene glycols. The assay was performed using a

modification of established methods [Stocks, *Analytical Biochem.*, 154:232-234 (1986)]. Samples were taken 30, 60, 90, 120, and 480 minutes after initiation of the conjugation reaction. Serial dilutions were made from each sample in a volume of 1.5 ml of 10 mM sodium phosphate buffer pH 7.4. Fluorescamine (0.3 mg/ml, Sigma Chemicals, St. Louis, MO) in acetone (0.5 ml) was added to each dilution while vortexing. Fluorescence of the samples was measured on a spectrofluorimeter (Photon Technology International, Monmouth Junction, NJ) with an excitation wavelength of 390 nm and emission at 475 nm. The resulting fluorescence is proportional to the concentration of free amino groups on the virus capsid. Standard curves were generated for each timepoint by plotting protein concentration versus fluorescent units. Degree of modification was obtained as the ratio between the slopes of conjugated and unconjugated viruses at similar timepoints.

TABLE 1

Degree of Modification for PEGylated AAV

Time (minutes)	TMPEG	CCPEG	SSPEG
0	0	0	0
15	95.8%	78.9%	100%
30	100%	85.25%	n.d.
60	n.d.	100%	n.d.

The reaction with CCPEG was complete after 60 minutes, a time when titer dropped by less than 10% and few aggregates had formed. Transduction efficiency of AAV was enhanced by conjugation with TMPEG over the entire reaction period. This reaction was complete in 30 minutes. Conjugation of AAV with SSPEG was complete within 15 minutes with minimal loss of titer over the entire reaction period.

Example 4 - Characterization of PEGylated AAV Preparations

Several physical tests were performed to confirm that activated PEG molecules were successfully conjugated to the AAV capsid.

A. Partitioning Assays

5 Partition coefficients of native and PEGylated viruses were determined as described previously [Delgado, *J. Biochem & Biophys. Meth.*, 29:237-250 (1994)]. Partitioning assays were performed at 25°C in single microfuge tubes containing 1g of a two-phase system of 4.75% (w/w) PEG 8000 (Sigma Chemicals, St. Louis, MO), and 4.75% Dextran T500 (Amersham Pharmacia
10 Biotech, Piscataway, NJ), in 0.15M NaCl containing 0.01 M sodium phosphate buffer, pH 6.8. The two phase system was prepared from stock solutions of 40% PEG, 20% dextran, 0.44M sodium phosphate buffer, pH 6.8 and 0.6M sodium chloride. AAV and PEGylated AAV were incorporated into the system by replacing 0.1 g of the water used to prepare the phases with 0.1g of virus in coupling buffer.
15 Samples were mixed 30-40 times by inversion and left to settle under gravity until complete separation of the phases was achieved. Aliquots from the top and bottom phases were analyzed for protein concentration by a microplate assay with Bio-Rad protein assay reagents and bovine serum albumin as a standard. The partition coefficient (K) is determined by the ratio between the protein concentrations in the
20 top and bottom phases.

TABLE 2

AAV Partition Coefficients for PEGylated AAV

AAV	Partition Coefficient
Unlabeled AAV	0.68
TMPEG	1.37
SSPEG	1.01
CCPEG	1.26

Partitioning of the viral preparations in an aqueous two-phase system

and calculation of partition coefficients (K) demonstrated that PEGylation modified the viral capsid significantly. K values shifted from 0.68 for unlabeled virus to 1.26 and 1.01 for CCPEG and SSPEG preparations, respectively. The TMPEG preparation demonstrated the highest level of conjugation with a K value of 1.37.

5 B. Zeta Potential

Zeta potential (electrophoretic mobility) of each PEGylated viral preparation was determined by laser Doppler anemometry (Zetasizer 3000, Malvern Instruments, Southboro, MA) in a thermostated microelectrophoresis cell as described previously at a 1:25 dilution in 10 mM potassium phosphate buffer pH 7.4.

10 Each reported value is the mean of three separate measurements.

The surface charge of the AAV capsid was effectively altered by the PEGylation process as zeta potential levels changed from -9.2 mV for native vector to -6.4 mV and -5.9 mV when conjugated to TMPEG and SSPEG, respectively.

The CCPEG preparation demonstrated the greatest shift toward neutrality to -5.1
15 mV.

 C. Electron Microscopy

Aliquots of conjugated viral particles were fixed in 1% glutaraldehyde, 0.05M cacodylate buffer pH 7.4 for 10 min at room temperature.

The solution was spread over 200-mesh carbon-coated grids (Electron Microscopy
20 Sciences, Fort Washington, PA): After 10 min excess suspension was wicked off grids with filter paper. The grids were stained with 2% uranyl acetate for 1 min, rinsed, and allowed to dry. Viral particles were viewed using a Philips CM-10 transmission electron microscope (Eindhoven, The Netherlands).

The average particle size for PEGylated AAV in three separate animal
25 studies is provided in the following table.

Table 3
PARTICLE SIZE DATA FOR PEGylated AAV

AAV	Average Radius (nm) for Formulations				
	Unlabeled starting material	TMPEG	SSPEG (99.9%)	CCPEG	MPEG unactivated PEG
W257 and W260	289.1±129.81 (72.1%)	634.4±102.3 (100%)	13.05±1.18	150.52±62.06 (77.8%)	---
	2922.7±240.31 (11.8%)		607.72±232.38 (0.1%)	2991.87±152.53 (4.1%)	
	5078.5±384.47 (16.1%)			5093.17±374.08 (7.3%)	
W259	262.8±117.42 (100%)	299.4±73.81 (100%)	17.08±3.47 (99.9%)	326.2±151.93 (90.4%)	---
			313.0±150.41 (0.1%)	5137.5±314.97 (9.6%)	
W267	135.2±5.84 (2.3%)	120.1±12.54 (84.5%)	17.64 ±4.25 (99.9%)	328.1 ± 140.77 (58.5%)	1003.6±201.2 (24.4%)
	1200.2±645.27 (90.1%)	703.1±307.46 (8.0%)	290.12±155.06 (0.1%)	2908.8±271.58 (20.2%)	1468.7±47.4 (9.6%)
	4820.1±647.96 (7.5%)	2863.2±262.86 (7.5%)		5069.0±390.66 (21.4%)	2908.9±289.86 (66.0%)

Example 5 - Neutralization Assays

In order to determine if the PEG-modification process of the invention (i.e., "pegylation") could preserve AAV transduction efficiency in the presence of neutralizing antibodies (NAB), conjugated AAV preparations were incubated in the presence of serum from C57BL/6 mice previously given multiple doses of AAV (1×10^{12} particles) or serum from non-immunized C57BL/6 mice.

Two hundred microliter aliquots (equivalent to a multiplicity of infection of 50) was applied to 84-31 cells (a 293-based E1/E4 complementing cell line) in triplicate. After a 2 hour incubation, vector is replaced with complete medium. Forty-eight hours after infection, cells are washed with PBS, fixed for 10 min in

0.5% glutaraldehyde, and washed twice with PBS containing 1 mM $MgCl_2$.

Transduction levels are reported as the ratio of cells transduced by virus incubated with immune serum to cells transduced by virus incubated with non-immune serum.

Transduction efficiencies of the PEGylated vectors were unaffected by the presence of immune serum at a 1:100 and 1:1000 dilution while that of the unconjugated preparation diminished by 49% and 32%, respectively. At the highest dilution, transduction efficiencies of PEGylated vectors fell by approximately 30% but were significantly higher than that of the unconjugated virus (Student's t-test, $p \leq 0.01$).

Mouse serum was analyzed for NAB by incubation at 56°C for 30 min to inactivate complement followed by dilution in DMEM in twofold increments starting from a 1:20 dilution. Each dilution (100 μ l) is mixed with virus, incubated for 1 hour at 37°C, and applied to 84-31 cells in 96-well plates (2×10^4 cells/well). After 1 hour at 37°C, 100 μ l of DMEM supplemented with 20% FBS was added to each well. Cells are incubated for 24 hours and the expression of green fluorescence protein (GFP) measured by FluoroImaging (Molecular Dynamics). NAB titers are calculated as the highest dilution at which 50% of the cells stained green.

Anti-AAV NAB was reduced following administration of TMPEG and SSPEG conjugated vector by 20% and 50% respectively. The CCPEG preparation was the least efficient vector for this application with transgene levels falling 10-fold below the other vectors at all timepoints and to undetectable levels 60 days after injection. NAB levels were only modestly reduced by this preparation.

Example 6 - Administration of PEG-Modified Vectors to Immunocompetent Animals

The following study demonstrates that PEGylation according to the present invention does not affect transduction efficiency of AAV when administered to the muscle or liver of immunocompetent animals.

C57BL/6 (H-2^b) mice (6 - 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). To determine the effect of PEGylation on

AAV transduction, unconjugated native AAV α 1AT or AAVCMVlacZ, the same viruses conjugated with various polyethylene glycols as described above and virus mixed with unactivated MPEG were administered either intramuscularly (5×10^{10} particles in 50 μ l PBS) or into the liver (1×10^{11} particles in 100 μ l PBS). Animals were necropsied 14 days post-injection and tissues divided with half rapidly immersed in peel-away molds containing OTC compound (Miles Diagnostics, Ekhart, IN) and flash frozen in a dry ice/isopentane bath for cryosectioning. Remaining tissue was placed in cold DMEM and processed for assay of expression as described below.

10 A. β -gal Assays

Treated tissues are excised from freshly euthanized animals and washed twice in cold PBS. When numerous samples are processed, excised tissues are stored no longer than 2 hours in cold DMEM. Tissues are rinsed in lysis buffer (provided with the β -gal ELISA kit, Boehringer-Mannheim) containing 4 mM Pefablock (Boehringer-Mannheim), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 μ g/ml pepstatin, 5 μ g/ml aprotinin (Sigma), 1 μ g/ml leupeptin, 0.5 mM EDTA, and 0.5 mM dithiothreitol (DTT). Tissues are homogenized in 1 ml of lysis buffer using a Brinkman polytron. Following homogenization, extracts are centrifuged at 14,000 rpm for 10 minutes. The protein concentration of the cleared supernatants was determined by a microplate assay with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in a dry ice/ethanol bath and stored at -80°C until assayed. β -gal concentrations are determined by an enzyme-linked immunosorbent assay (ELISA) using a β -gal ELISA kit (Boehringer-Mannheim) according to manufacturer's instructions.

25 After intramuscular injection of an AAVCMVlacZ vector, initial transduction efficiency of the TMPEG, SSPEG and unconjugated virions were not statistically significant (Student's t-test, $p \leq 0.05$); in each case transgene expression was generally stable during the period of the experiment. The CCPEG preparation was the least efficient and produced transgene levels that were reduced 10-fold below the other vectors at each time point, with gene expression dropping to

undetectable levels 60 days after injection. All animals that received intramuscular injection of either the native or the conjugated preparations developed high levels of neutralizing antibody against AAV capsid proteins.

B. Human Alpha-1 Antitrypsin Assay

5 Concentration of human α 1AT in mouse serum was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [W.D. Xiao et al, *J. Virol.*, 73:3994-4003 (1999)]. Microtiter plates (MaxiSorp, Nunc) were coated with 100 μ l rabbit anti-human α 1AT antibody (1:100, Sigma) at 4°C overnight, blocked and incubated with standards or samples overnight at 4°C. After
10 washing, the primary antibody (1:1000, goat anti-human α 1AT, Sigma) was incubated with the captured antigen for two hours at room temperature. Secondary antibody (anti-goat IgG-peroxidase conjugate, Sigma, 1:10,000) was added for 2 hours at room temperature. ABTS reagent (Boehringer Mannheim) was added and concentration determined from absorbance readings at 405 nm. The sensitivity of
15 the assay is 0.3 to 30 ng/ml.

When animals were injected intravenously with an AAV α 1AT vector, TMPEG and SSPEG preparations only differed from the native vector by the rate at which gene expression plateaued. A peak level of gene expression equal to 1×10^4 ng/ml of α 1AT was noted for native vector as well as SSPEG and TMPEG
20 conjugated vector when evaluated 14 days after injection.

Example 7 - Analysis of AAV-specific Immunoglobulins

Serum samples from mice were assessed for AAV-specific isotype specific immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, IgM) by ELISA as described previously [N. Chirmule, et al, *J. Immunol.*, 163:448-455 (1999)]. Microtiter plates
25 (MaxiSorp, Nunc) were coated with 100 μ l AAV antigen (5×10^{10} particles/ml) in 0.1 bicarbonate buffer, pH 9.6 overnight at 4°C, washed four times with PBS containing 0.05% Tween 20 and blocked in PBS containing 3% BSA for three hours at room temperature. Serum samples at a 1:100 dilution were added to the antigen-coated plates and incubated overnight at 4°C. Plates were washed four times with

PBS containing 0.05% Tween 20 and incubated with biotin conjugated rat anti-mouse anti-IgG1, IgG2a, IgG2b, IgG3, IgM (PharMingen, San Diego, CA) at a 1:1000 dilution for three hours at room temperature. Plates were washed as above and a 1:10,000 dilution of alkaline phosphatase conjugated avidin (Sigma Chemical Company, St. Louis, MO) was added for two hours at room temperature. After four washings, p-nitrophenyl phosphate in diethanolamine buffer was added (Sigma Chemical Company, St. Louis, MO) and optical densities read at 405 nm on a microplate reader (Dynatech Laboratories, Chantilly, VA).

Characterization of the anti-AAV antibody isotypes revealed this was the case as the CCPEG preparation was the only formulation to significantly enhance production of IgG1 antibodies, those associated with the Th2 pathway.

Example 8 - Immunization Experiments

Results from immunization studies using rAAV which has been pegylated according to the present invention demonstrates that the pegylated rAAV of the invention permit enhanced gene expression.

A. PEGylation Allows for Significant Gene Expression In Immunocompetent Animals After Prior Exposure to the Native Vector

As shown herein, transduction efficiency of some PEGylated vectors is not affected by the presence of neutralizing antibodies (NABs) to native vector *in vitro*. In order to determine if this effect could also occur *in vivo*, animals were challenged with a second dose of PEGylated vector (1×10^{11} particles i.v. or 5×10^{10} particles i.m.) 30 days after initial immunization with the same dose of native AAV. The second vector was AAVCMVlacZ for intramuscular injections and AAV α 1AT for intravenous infusions. Animals that received two doses of native vector intramuscularly did not demonstrate significant levels of gene expression upon rechallenge. Animals that received the TMPEG preparation had significant levels of gene transduction upon readministration, although it was reduced approximately 7.5-fold as compared to naïve animals. The level of gene expression, obtained with SSPEG preparation was substantial but 10-fold lower than naïve

animals. A significant level of gene expression was also seen with the CCPEG preparation, but this was the least effective.

Administration of two doses of native AAV intravenously failed to produce significant levels of gene expression following the second vector. All PEGylated preparations delivered intravenously yielded high levels of gene transfer following intravenous immunization with native vector that were similar to that observed in naïve animals ranging from 100% of naïve for TMPEG to 40% of native for CCPEG.

B. PEGylation Allows for Significant Gene Expression by Unconjugated AAV In Immunocompetent Animals After Prior Exposure to PEGylated Virus

The impact of humoral immune responses to PEGylated vectors on gene transfer with native virus was studied. Mice immunized with different preparations of PEGylated AAV were given a second intramuscular injection of 5×10^{10} particles of the native vector. Significant levels of gene expression were detected in animals immunized with CCPEG and SSPEG preparations, despite the presence of anti-AAV antibodies. Mice immunized with the TMPEG preparation produced the most significant levels of gene expression upon rechallenge with native virus, but were still somewhat below those seen in naïve animals.

Native virus directed to liver produced significant levels of gene expression in animals immunized intravenously with PEGylated AAV. Mice immunized with SSPEG and CCPEG preparations demonstrated significant levels of gene expression upon readministration, as measured by α 1AT serum levels, that were only 42% (SSPEG) and 21% (CCPEG) lower than that seen from a single dose of vector administered to naïve animals. Native vector produced the most significant level of gene expression in animals immunized with TMPEG despite the fact that those animals had the highest levels of NAB to native vector prior to administration.

C. Repeated Administration of PEGylated AAV Produces Significant Levels of Gene Expression in Liver but Not in Muscle

C57BL/6 received two consecutive intramuscular doses of PEGylated AAV. The goal of these experiments were to evaluate gene transfer efficiency with a particular form of PEGylated AAV following a previous immunization with the same type of PEGylated vector. Readministration of the same type of PEGylated vector was substantially reduced when compared to an intramuscular injection into naïve animals for each PEGylated vector ranging from a 20-fold reduction with CCPEG, to a 200-fold reduction with SSPEG. All animals developed significant titers of anti-AAV NAB. When administered intravenously, gene expression after two doses of the CCPEG preparation produced α 1AT serum levels equivalent to those seen in naïve animals, despite the presence of anti-AAV NAB. The SSPEG preparation produced slightly reduced gene expression after readministration (i.e., 50% of naïve animals). Transduction of the TMPEG preparation upon readministration surpassed that of naïve animals.

Example 9 - Production of Conjugated Adenoviral Vectors

E1/E3-deleted adenoviral vectors expressing β -galactosidase (H5.010.CMV.LacZ) were used for these studies and were amplified in 293 cells using a modification of established methods and purified from cell lysates by banding twice on CsCl gradients (Graham and Van der, *Virology*, 52:456-467 (1973)). Aliquots of virus were desalted on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) and equilibrated with the respective buffer for optimal conjugation (see below). Viral concentrations were determined by UV spectrophotometric analysis at 260 nm. Transduction titer (i.e., LacZ forming units or lfu) was determined by limiting dilution infections of 293 cells. The particle/lfu ratio of both conjugated and unconjugated virus was approximately 100. Protein content of Ad preparations was determined by a micoplate assay with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard.

These adenoviral vectors were conjugated with the three types of activated PEG as described in Example 1B.

Example 10 - Conjugation of activated MPEGs to adenoviral capsids occurs rapidly with minimal loss of viral infectivity

5 The TMPEG-adenovirus (Ad) preparation retained viral titer over the entire reaction period while the SSPEG preparation lost 70% of the original titer when the conjugation reaction was complete. Premature termination of the SSPEG reaction (i.e., at 75 minutes when it was 70% complete) resulted in a preparation with 90% residual activity. Modification of the virus with CCPEG was complete in 90
10 minutes, however, only 11% of the original infectivity remained. Fractionation of this preparation over a Sephadex G50 column produced distinct viral populations. The first peak represented PEGylated monomer virus with high infectivity while the second peak contained aggregated virus (per electron microscopy and static laser light scatter, data not shown) with low activity. Only the first peak was isolated and
15 used in additional studies. Addition of unactivated MPEG to Ad preparations had no effect on infectivity as compared to vector in buffer alone (data not shown).

 Significant loss of titer with CCPEG can be attributed to the production of large viral aggregates due to extensive cross-linking of the polymer with viral capsids. This is manufactured as a mixture of two isoforms, one that will attach to
20 proteins by a single amide bond, and another that is capable of forming two amide bonds with the target protein. Once aggregates were removed, the remaining viral suspension proved to be highly infectious and extremely stable under various storage conditions. The SSPEG preparation also experienced a significant drop in titer after conjugation for one hour at room temperature. No aggregation phenomena was
25 detected with this compound, however, loss in titer could be attributed to attachment of multiple SSPEG molecules to a single lysine residue. In order to maintain substantial viral infectivity with significant modification, a reaction time of 75 minutes, which modified 70% of available lysine residues and maintained titer was selected. While this was the only protocol that did not completely modify the viral

capsid, the resultant vector could efficiently evade neutralization by immune serum and maintained the highest titers at the various storage conditions with respect to the other PEGylated preparations. These data indicate that complete modification of the viral capsid with this polymer is not a strict requirement for production of highly efficient viral vectors.

Example 11 - Characterization of Ad-PEG Complexes

Several physical tests were performed to confirm that activated PEG molecules were successfully conjugated to the adenovirus capsid. Partitioning assays and zeta potential were performed as described in Example 4.

Partitioning of the viral preparations in an aqueous two-phase system and calculation of partition coefficients (K) demonstrated that the viral capsid was significantly modified. K values shifted from 0.7 for unlabeled virus to 1.76 and 1.96 for TMPEG and SSPEG preparations, respectively. The CCPEG preparation demonstrated the highest level of conjugation with a K value of 3.56.

Zeta potential analysis revealed that surface charge of the adenovirus capsid significantly changed from -48.1 mV to -27.8 and -24.2 mV when conjugated to TMPEG and SSPEG respectively. The CCPEG preparation demonstrated the greatest shift toward neutrality to -16.2 mV.

Example 12 - Adenovirus-PEG Complexes are Protected from Neutralization by Immune Serum

PEGylated preparations were added to HeLa cells in the presence of neutralizing antibodies to adenovirus capsid proteins and transduction levels were compared to that of the same preparation incubated in non-immune serum. This study was performed essentially as described in Example 5 above, with the exception that the immune serum was derived from C57BL/6 mice harvested 28 days after intravenous injection of adenovirus vector.

Transduction efficiency of the unconjugated virus was significantly reduced by neutralizing antibodies. The TMPEG preparation did shield the virus partially

from neutralization while transduction of the SSPEG and CCPEG preparations was unaffected by the presence of neutralizing antibody.

Example 13 - Stability of PEGylated Adenoviruses

As shown in the study below, MPEG, when added at a concentration similar
5 to that employed in the PEGylation reaction, extended viral stability at 42°C and 25°C beyond that of the unconjugated adenovirus, but viral titers were substantially lower than those of the PEGylated virions. Precipitates were eventually detected in these preparations which significantly contributed to losses in transduction. However, addition of this excipient to adenovirus formulations at 4 °C significantly
10 enhanced stability beyond that of the native virus for up to five months due to its ability to maintain capsid assembly at this temperature. Addition of glycerol to PEGylated preparations did not affect the stability of the virus at -20 °C. This result is encouraging as glycerol (and its associated toxicity) can be eliminated from preparations for clinical use. While each PEGylated preparation lost about 1 log of
15 titer over four months, they were stored in the absence of any additional cryoprotectants. Addition of carbohydrates, surfactants and other stabilizers will enhance stability and reduce loss in titer to negligible levels.

A. PEGylated Adenoviruses are Significantly More Stable Under Various Storage Conditions than Unconjugated Virus

20 The ability of PEGylation to stabilize virus at a wide variety of temperatures was assessed. Preparations were stored in potassium buffered saline (KPBS) with or without the addition of 10% glycerol at -20°C and at 4°C. Unconjugated adenovirus suffered a drop in titer of one log after storage at 4°C for eight hours, falling to undetectable levels seven days after storage. This rapid
25 degradation of viral capsids is readily detected by electron microscopy. After 24 hours at 4°C, the unconjugated viral preparation consists mainly of single viral capsid proteins, mostly hexons; only a few intact viral particles could be detected. The CCPEG preparation was the least stable of the remaining preparations as titer fell one log after incubation at 4°C for 24 hours. Electron micrographs display
30 unequivocal evidence that this preparation is significantly more stable than

unconjugated virus as photos taken at twelve days after storage reveal intact virions. TMPEG preparations demonstrated a negligible loss in titer at 4°C for one week; after 42 days, titer fell by two logs and remained at this level for the duration of the study. At this time, the preparation mainly consisted of intact viral capsids with only a few compromised virions. The SSPEG preparation underwent an initial drop in titer of one log after eight hours at 4 °C and maintained titer for up to 150 days. This preparation was indistinguishable from freshly purified virions upon inspection by electron microscopy 120 days after storage at 4°C.

Stability studies of PEGylated preparations at -20°C revealed that the addition of glycerol was not necessary for maintenance of titer. In the absence of glycerol, titers of unconjugated virions dropped 5.5 logs after 15 days at -20°C in KPBS. Degradation of the virus steadily continued at the rate of 1 log/month for the remainder of the study. Addition of 10% glycerol significantly enhanced stability as only one log unit of titer was lost in the unconjugated preparations stored at -20°C over four months. SSPEG conjugated virions were the most stable of all the preparations studied with a drop in titer of 0.8 log over 120 days at -20°C; addition of glycerol only slightly enhanced stability. Titer of the TMPEG preparation was the most sensitive to the addition of glycerol. The preparation without glycerol demonstrated a drop of one log after 90 days in storage, while the titer of the preparation that contained glycerol dropped only 0.5 log unit. CCPEG virions were least stable with a 4 log decrease of infectious virus after 90 days of storage at -20°C; glycerol did not help.

B. PEGylation Enhances Adenoviral Stability under Extreme Storage Conditions

Even though the stability data for PEGylated viral preparations at 4°C and -20°C was very encouraging, these conditions would still require shipment on ice to various clinical sites. In order to assess the possibility of vector shipment under ambient conditions, samples were stored in KPBS at 25°C and at 42°C. Upon storage at 25°C, unconjugated adenovirus dropped in titer at a rate of 1 log/day. Addition of monomethoxy poly(ethylene) glycol to the adenovirus preparation

slightly enhanced stability of unconjugated adenoviral preparations with the loss of titer at an average rate of 0.6 log units/day.

CCPEG preparations were very stable at room temperature. An initial drop in titer from 5.84×10^{10} lfu/ml to 3.18×10^{10} lfu/ml was detected after six hours, which subsequently stabilized for one week. TMPEG preparations were stable for 24 hours while titer of the SSPEG preparation remained constant for up to five days, which would allow for priority shipment of vector in the absence of dry ice. When stored at 42°C, titer of unconjugated adenovirus preparations initially fell at a steady rate of approximately 1 log every 45 minutes. Addition of inactivated MPEG delayed the loss, which did eventually diminish two logs over three hours. TMPEG and CCPEG preparations demonstrated similar degradation rates with titers falling 1.5 log units over the study period. The SSPEG preparation was the most stable with negligible loss of titer for 18 hours at this condition. The SSPEG preparation demonstrated a negligible loss of titer at 42°C for up to eight hours. Thus, this preparation could survive exposure to extreme temperatures upon shipping without significant loss of titer.

Example 14 - Administration of PEGylated Adenoviral Vectors to

Immunocompetent Animals

C57BL/6 (H-2^b) mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). Preparations were administered either via the tail vein (1×10^{11} particles in 100 µl KPBS) or intratracheally (5×10^{10} particles in 50 µl KPBS). Animals were necropsied four days later and excised tissues washed twice in cold PBS and stored in cold DMEM for processing. Tissues were homogenized in 1 ml of lysis buffer using a Brinkman polytron. Extracts were centrifuged at 14,000 rpm for ten minutes. Protein concentration of supernatants was determined with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in dry ice and stored at -80°C until assayed. β-gal concentrations were determined with a β-gal ELISA kit (Boehringer-Mannheim) according to manufacturer's instructions.

When administered to the lung, unconjugated virus produced $1.42 \times 10^4 \pm 3.6 \times 10^3$ pg of β -galactosidase/mg protein. The CCPEG and SSPEG preparations produced β -galactosidase expression levels two fold higher than unconjugated virus. The TMPEG preparation demonstrated the largest increase in transduction with a 2.5-fold increase in beta-galactosidase. Addition of inactivated MPEG to Ad preparations raised transduction to a level three times that of the unconjugated virus.

Administration of unconjugated adenovirus intravenously yielded beta-galactosidase levels of $3.0 \times 10^6 \pm 1.1 \times 10^5$ pg /mg of protein in the liver. *In vivo* transduction of liver was 6-fold higher with TMPEG, CCPEG and SSPEG. Addition of MPEG to the viral preparation slightly raised transduction levels beyond that of the unconjugated virus.

PEGylation of adenoviral vectors enhanced transduction efficiency when administered intratracheally or intravenously. This effect was somewhat unexpected as the majority of lysine residues that are present on the viral capsid are concentrated on the fiber and penton proteins which are necessary for viral binding and entry into target cells [(Adam, *et al.*, Acta Microbiologica Academiae Scientiarum Hungaricae, 24:181-187 (1977), Bergelson, *et al.*, Science, 275:1320-1323 (1997), Wickham, *et al.*, Cell, 73:309-319 (1993)]. However, the new physical characteristics of the PEGylated viruses may contribute to the observed increase in viral transduction. Zeta potential measurements have shown that the adenovirus bears a significant negative charge on the capsid. Surface charge of viral vectors can significantly affect the level of transduction in various target tissues (Fasbender, *et al.*, J. Biol. Chem., 272:6479-6489 (1997)). It has been found that adenoviral transduction is inhibited due to static repulsion between the negatively charged sialic acid residues on the cell surface and the virus (Arcasoy, *et al.*, Am. J. Resp. Cell & Molec. Biol., 17:422-435 (1997)). PEGylation effectively masks the groups responsible for this charge, producing an environment that would favor non-specific interaction of the virus with the cell membrane. Particle size measurements of the final PEGylated preparations also revealed that each method produced a suspension of single viral particles which enhance the number of virions that come in contact with cell

monolayers and, as a result, can increase transduction efficiency. Partition coefficients for the PEGylated virions indicate that the modified vectors have an increased affinity for hydrophobic environments that would allow for an increased ability to indiscreetly partition through cell membranes. Initial studies to assess the mechanism by which transduction efficiency of PEGylated adenoviruses is enhanced supports this theory as permeability of the PEGylated vectors across differentiated monolayers is significantly enhanced (data not shown).

Example 15 - Assessment of Formulations for Use in Frozen and Lyophilized Viral Preparations

Two buffers that are acceptable for human use, 10 mM sodium phosphate (DPBS) and 10 mM potassium phosphate (KPBS) buffered saline, were assessed for their ability to maintain pH at low temperature.

A. Effect of Temperature on Various Formulations

Materials: Sucrose USP, beta cyclodextrin, D-mannitol USP, D(+) trehalose, sorbitan monolaurate (Span 20), and phosphate-buffered saline (PBS) were purchased from Sigma (St. Louis, MO). Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and potassium chloride USP were purchased from JT Baker (Phillipsburg, NJ). Glycerol USP was purchased from EM Science (Gibbstown, NJ). Tertiary amine beta cyclodextrin was purchased from Cerestar USA, Inc. (Hammond IN). Pluronic block copolymer F68 was kindly provided by the BASF corporation (Mt. Olive, NJ). Universal pH Indicator was purchased from Fisher Scientific (Pittsburgh, PA).

Sample Preparation: All formulations were prepared under GLP conditions and sterilized by filtration through a 0.22 μm filter (Corning). For all studies, 250-1000 μl aliquots were added to autoclaved 3ml clear borosilicate glass vials (Wheaton, Millville, NJ). Vials were topped with 13 mm gray butyl rubber stoppers (Wheaton) and sealed with tear-off aluminum seals (Wheaton). For pH studies, 10 μl of Universal pH Indicator was added to monitor pH changes during freezing.

pH Studies: Eight samples were prepared for each formulation as described. Four samples contained Universal pH indicator [M.A. Croyle et al, Pharm. Dev. Technol., 3(3):373-383 (1998); S. Nema and K.E. Avis, J. Parenteral Science & Technology, 47(2):76-83 (1993)]. The remaining samples did not contain the indicator and were used to determine viral titer after freezing. Samples were frozen for 12 hours at -20°C and -80°C . pH of the frozen products were determined visually and noted for each formulation. Samples not containing indicator were slowly warmed to 25°C and assessed for viral potency. The results are shown in Table 4 below.

10

Table 4

Formulation	<u>KPBS (pH)</u>		<u>DPBS (pH)</u>	
	<u>-20°C</u>	<u>-80°C</u>	<u>-20°C</u>	<u>-80°C</u>
Buffer alone	6.0	4.5	5.0	4.0
Glycerol	6.0	6.5	5.0	5.0
15 0.25 M Sucrose	6.0	4.0	5.0	4.0
0.5 M Sucrose	6.0	4.0	5.5	4.0
1.0 M Sucrose	6.5	5.5	6.0	4.0
0.25 M Trehalose	6.0	4.0	5.5	4.0
0.5 M Trehalose	6.0	4.0	5.5	4.0
20 1.0 M Trehalose	6.0	5.0	6.0	4.0
0.5% BCD	6.5	6.0	5.5	5.5
5% TMBCD	7.4	7.4	7.4	7.4

20

pH of sodium phosphate buffered saline dropped from pH 7.4 to 5.0 when frozen at -20°C and to pH 4 at -80°C (see Table above). Addition of glycerol to the buffer did not prevent the drop in pH upon freezing. Differential scanning calorimetry demonstrated that this change in pH is associated with precipitation of buffer components during freezing. Addition of sucrose or trehalose to the buffer could not prevent this phenomenon at -80°C , but each sugar at a 1M concentration did prevent dramatic pH changes at -20°C . Potassium phosphate buffer did not demonstrate the significant changes in pH as seen with the sodium phosphate buffer

30

(DPBS) at -20°C , but did experience similar drops in pH at -80°C . Addition of cryoprotectants did not significantly affect the final pH of the preparation. However, it is important to note that for each buffer system, tertiary amine beta cyclodextrin (TMBCD) could maintain physiological pH upon freezing.

5 B. Effect of Temperature on Various Formulation Containing Viral Vectors

Virus was formulated in the following manner and frozen at the respective temperature for 12 hours. Samples were thawed at 25°C and viral titer assessed in order to determine if change in pH would significantly affect physical stability.

10 As used throughout the following examples, the term lac-forming unit (lfu) defines the number of infectious viral particles present in a preparation of beta-galactosidase (lacZ) expressing virus as measured by limiting dilution, infection of 293 cells [or 84-31 cells, a cell line expressing adenovirus E1 and E4 in the case of AAV (K.J. Fisher et al, *L. Virol.*, 70:520-532 (1996))], histochemical staining and visual identification of lac⁺ cells. Samples were serially diluted in DMEM supplemented with 2% fetal bovine serum. Medium was removed from 12 well plates seeded with 1.5×10^4 cells/well and 0.2 ml of the appropriate dilution was placed on the monolayers. After two hours at 37°C , 2 ml of complete medium was added to each well and the infection continued for 16 hours at 37°C . At this time, medium was removed and cells stained for beta-galactosidase expression as described previously [M.A. Croyle, et al, *Pharm. Dev. Technol.*, 3(3):365-372 (1998)]. Lac⁺ cells were tallied from a minimum of 20 microscope fields (approximately 48,000 cells). Lac-forming units were calculated as described previously [M.A. Croyle et al, *Pharm Dev Technol*, 3(3):373-383 (1998)]. The sensitivity of the assay was 10 to 1×10^{12} lfu/ml.

1. Preparation of Adenovirus

First-generation adenovirus expressing *E. coli* β -galactosidase under the control of a CMV promoter was amplified in 293 cells using a modification of established methods [F. L. Graham and A.J. van der Eb, *Virology*,

52:456-467 (1973)]. Virus was purified from cell lysates by banding twice on CsCl gradients followed by desalting on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) equilibrated with each respective formulation. Concentration of the virus was determined by UV spectrophotometric analysis at 260 nm and lac-forming assays. All experiments were performed with freshly purified adenovirus.

2. Preparation of Adeno-Associated Virus

Production of recombinant AAV2 expressing the E. coli beta-galactosidase gene under the control of a CMV promoter involved transfecting 60% confluent 293 cells in 150 mm dishes with a 1:1:2 ratio of *cis* plasmid (pAAVlacZ, with AAV ITRs), *trans* plasmid (p5E18, containing AAV2 *Rep* and AAV1 *Cap* [W. Xiao et al, *J. Virol.*, 73(5):3994-4003 (1999)]) and helper plasmid (p Δ 13, an adenovirus plasmid with most of the late genes deleted and an 8 kb deletion in the E2b region). Transfection was performed by calcium phosphate precipitation. Ninety-six hours after transfection, cells were harvested and subjected to two rounds of CsCl gradient purification as described previously [K. J. Fisher et al, *Nature Medicine*, 3:306-312 (1997)]. Virus was desalted by dialysis against respective formulations.

3. Results

Adenoviral preparations were quite sensitive to pH changes upon freezing (Figure 1A). Preparations that maintained initial pH (7.4) did not experience significant drops in titer upon thawing. Preparations that underwent a drop of 3 pH units suffered a one log loss of titer. Adeno-associated virus (AAV) was not as sensitive to pH changes upon freezing as the adenovirus (Figure 1B). AAV preparations that experienced a drop of 3 pH units upon freezing lost 0.5 log of infectious virus. As stated above, potassium phosphate buffered saline maintained pH to a higher degree than sodium phosphate buffered saline when frozen at -20 °C. Adenovirus stored in 10% glycerol in this buffer system demonstrated superior stability over sodium phosphate buffered preparations. As a result, potassium

phosphate buffered saline was the buffer of choice for all formulations unless otherwise indicated.

Example 16 - Stability of Adenoviral Formulations at -20°C and 4°C.

After screening potential formulations and buffer systems for the ability to maintain pH at low temperature, large scale preparations of formulated virus were stored at -20 and 4°C for a period of two years. Prior to storage, viral titer was determined by lac-forming assay (t=0). In order to assess the real-time stability of vector in each formulation, 4-6 vials from each lot were removed and assessed for transduction efficiency. Viral titer of each formulation was compared to titer from virus produced in the same lot stored at -20°C in KPBS. Expiration dates were assigned to each preparation at the time when titer fell to 90% of its original value (i.e. suffered 10% loss (0.1 log)) as is standard practice in the pharmaceutical industry [Carstensen, J.T., Drug Stability: Principles and Practices. Second Edition, Drugs and the Pharmaceutical Sciences. Vol. 68. 1995, New York, New York: Marcel Dekker, Inc.; International Conference on Harmonization (ICH), Harmonized Tripartite Guideline, Stability Testing of New Drug Substances and Products, 1993; Center for Drugs and Biologics FDA, Guidelines (1987): Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics, 1987: Rockville, MD].

Table 5. Expiration Dates for Adenoviral Formulations Stored at -20 °C.

	<u>Formulation</u>	<u>Expiration</u>
	1M Sucrose/KPBS/5% TMBCD	690 days
	1M Sucrose/KPBS/0.5% BCD	360 days
	1M Sucrose/KPBS	70 days
25	1M Sucrose/DPBS/5% TMBCD	45 days
	0.5 M Sucrose/KPBS	18 days
	10% Glycerol/KPBS	12 days
	10% Glycerol/DPBS	2 days
	DPBS	12 hours

Glycerol preparations were the least stable at -20°C . The sodium phosphate buffered preparation lost 10% of the original titer in 2 days (Table 5). Potassium phosphate buffer extended the shelf life of the virus to 12 days. When glycerol was removed and sucrose added to the preparation, titer did not drop for approximately 1 month. Addition of cyclodextrins to the formulation extended shelf life to 360 (beta cyclodextrin)- and 690 (tertiary amine beta cyclodextrin) days which is significantly longer than that of virus in glycerol at -80°C (~ 37 days, unreported observation).

Table 6. Expiration Dates for Adenoviral Formulations Stored at 4°C .

	<u>Formulation</u>	<u>Expiration Date*</u>
10	0.4% Sucrose/0.4% Mannitol/ 0.001% Span 20	35 days
	1M Sucrose/KPBS/BCD	20 days
	1M Sucrose/KPBS	10 days
	1M Sucrose/DPBS	8 days
	0.5 M Sucrose/KPBS	7 days
15	0.25M Sucrose/KPBS	2 days
	DPBS	15 minutes

***Note:** Expiration dates represent the time for a preparation to lose 10% (0.1 log) of the original titer.

All formulations stored at 4°C significantly enhanced viral stability (Table 2). Sucrose formulations containing beta cyclodextrin and surfactant did not display significant drops in titer for approximately one month.

Example 17 - Stability of AAV Formulations

Initial stability studies with adeno-associated virus demonstrated that this virus is significantly more stable than the adenovirus.

Table 7. Expiration Dates for Adeno-Associated Virus Formulations.

Formulation	-80°C	-20°C	4°C	25°C
0.4% Sucrose				
0.4% Mannitol	26.7 days	89.5 days	240 days	24.7 days
5 0.01% Pluronic				
Protamine				
(0.5 mg/ml)				
0.4% Sucrose				
0.4% Mannitol	149.4 days	149.65 days	150.1 days	153.5 days
10 0.001%Span 20				
Protamine				
(1.0 mg/ml)				
DPBS	32.4 days	37.4 days	n/d	n/d
10% glycerol				
15 DPBS	27 days	n/d	135.4 days	125.5 days

***Note:** Expiration dates represent the time for the preparation to lose 10% (0.1 log) of the original titer. n/d – stability not assessed at this temperature.

It took approximately 4 months for a preparation stored at 4 °C in sodium phosphate buffered saline to lose 0.1 log of infectious virus (Table 7). Similar results were seen at 25°C. A formulation consisting of sucrose, mannitol, Span 20 and protamine extended AAV stability to 5 months at 4 and 25°C. Similar expiration dates were also noted with this formulation at -20 and -80°C.

Example 18 - Effect of Formulation on Transduction Efficiency *In Vivo*

Because adenoviral vectors are suitable for many gene therapy applications, we studied the effect of these formulations on transduction efficiency after intramuscular, intravenous and intratracheal injection in C57BL/6 mice. Transduction efficiency of formulated virus was compared to vector in phosphate buffered saline and the standard 2% glycerol preparation commonly used in pre-clinical testing of viral vectors [F. Borellini and J.M. Ostrove, "The Transfer of Technology from the Laboratory to the Clinic: In Process Controls and Final

Product Testing", in Gene Therapy Technologies, Applications and Regulations, A. Meager, Editor. 1999, John Wiley & Sons: West Sussex, England. p. 359-373]. A 5% glycerol formulation was also included as a semi-toxic control by which to compare our formulations.

5 A. *In Vivo* Testing of Formulated Virus

C57BL/6 mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). Preparations were administered either via the tail vein (1×10^{11} particles in 100 μ l formulation), intratrachially (5×10^{10} particles in 50 μ l), or intramuscularly (5×10^{10} particles in 50 μ l). Four animals from each group were necropsied either 4 days (intravenous and intratracheal) or 7 days (intramuscular) after injection. Tissues were homogenized in 1 ml of lysis buffer using a Brinkman polytron. Extracts were centrifuged at 14,000 rpm for 10 minutes. Protein concentration of supernatants was determined with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in dry ice and stored at -80°C until assayed. β -gal concentrations were determined with a β -gal ELISA kit (Boehringer-Mannheim) according to manufacturer's instructions. Blood samples were collected from remaining animals of each group via the retro orbital sinus 4 and 7 days after injection for assessment of liver function by an outside contract lab (Antech, New York, NY). All animals were bled prior to initiation of the study for assessment of baseline LFT.

15 B. Results

A significant increase in transgene expression was detected after intramuscular injection of the sucrose/beta cyclodextrin and 5% glycerol formulations. The other formulations did not effect gene expression in muscle and were well tolerated. While the sucrose/tertiary amine beta cyclodextrin formulation enhanced transduction efficiency of the virus in the lung, transduction efficiency was reduced by the 5% glycerol preparation. Signs of acute inflammation were detected in lungs from mice given this preparation (data not shown). This effect was not detected in animals given the other formulations. All formulations tested slightly enhanced transduction efficiency when administered intravenously. The 5%

glycerol preparation produced a marked increase in SGOT/SGTP levels beyond that routinely observed after administration of vector. This was not seen with the other formulations.

Example 19 - Development of Lyophilized Viral Vectors

5 Even though the formulation efforts described herein significantly enhanced adenoviral stability at -20 and 4°C, they would still require shipment on ice. Lyophilization, a technique commonly employed to extend the chemical and physical stability of labile compounds at ambient temperatures, was considered as a practical alternative. The lyophilization process consists of three stages: freezing,
10 primary drying and secondary drying. During the freezing step, samples are frozen in the range of -40 to -50°C. When the product is completely frozen, the pressure in the lyophilization chamber is reduced and heat is applied to the product. Under these conditions, water is removed by the sublimation, a phase change from the solid state directly to the vapor state without the appearance of an intermediate liquid
15 phase. As freeze-drying proceeds, the thickness of the frozen layer decreases. This is the primary drying phase. After primary drying, additional drying is necessary to remove any water that remains in the product. During secondary drying, heat is slowly added to the highest allowable temperature to maintain product viability and maintained at this level until the process is complete.

20 A large batch of adenovirus was purified and divided into five separate groups. Each group was desalted into different formulations. Excipients were selected according to their ability replace water and form a protective shell around the viral capsid in order to maintain protein conformation during the freezing and drying process, as described by Timasheff, Carpenter and others [Carpenter, J.F. and
25 J.H. Crowe, Cryobiology, 25: 244-255 (1988); Crowe, J.H., *et al.*, Cryobiology, 27: 219-231 (1990); Carpenter, J.F. and J.H. Crowe, Biochemistry, 28: 3916-3922 (1989); Timasheff, S.N., Annu Rev Biophys Biomol Struct., 22: 67-97 (1993)]. The initial titer of the lot was 1×10^{11} lfu/ml. One milliliter aliquots were placed in glass vials and lyophilized under a standard protocol (-40°C for 2 hours, -33°C for 12

hours, -25°C for 5 hours, 10°C for 4 hours and 20°C for 8 hours at a pressure of 30 mtorr).

For each lyophilization study, a lot of virus was divided into two groups with one half desalted in the desired formulation and the other desalted in KPBS containing 10% glycerol. The latter preparation was stored at -20 °C and served as a control by which to assess the ability of lyophilization to enhance viral stability. Viral titers were assessed prior to storage at -20 °C and lyophilization (t=0). Vials containing the formulated preparation were placed in a single shelf research grade lyophilizer (FTS Systems, Stone Ridge, NY) and cooled at a rate of 1 °C/min to -40°C. Three vials of formulation without virus were fitted with lead platinum RTD temperature probes (FTS) and placed in the front, middle and back of the lyophilization unit to monitor product temperature changes during the process. After cooling, all samples were lyophilized according to methods tailored to the specific formulation under study [M. J. Pikal, Freeze-Drying of Proteins, Part 1: Process Design, Biopharm., 1990, Sept: p. 18-27]. After drying was complete, samples were stoppered under vacuum and sealed with 13 mm tear-off aluminum seals (Wheaton). Samples were stored at 4°C until assayed for viral titer. Lyophilized virus was reconstituted with 1 ml sterile water for injection (USP) and assayed with samples stored at -20°C in KPBS and 10% glycerol.

Preparations lyophilized in sodium or potassium phosphate buffer alone suffered significant losses of 5 and 4 logs of infectious virus respectively (Figure 2). When sucrose, mannitol and Span 20 were added to formulations, titer fell by approximately 1 log. Titer fell by less than 1 log in preparations lyophilized in 1M sucrose.

Example 20 - Effect of Viral Concentration on Glass Transition Temperature (Tg') of Formulations for Lyophilization

Even though all excipients significantly improved recovery of adenovirus after lyophilization, each preparation still suffered a drop in titer, which was attributed to the process itself. Thus, formulations were further characterized in

order to maximize viral recovery upon completion of the lyophilization cycle. One parameter that is important for ascertaining the required processing conditions is the glass transition temperature (T_g'), the temperature of maximum freeze concentration where, in a frozen solution, the residual non-ice phase forms a glass [F. Franks, *Cryo-Letters*, 11:93-100 (1990)]. If primary drying is carried out above T_g' , the freeze concentrate behaves as a viscous liquid, the microstructure of ice formed upon freezing will be lost and product recovery is significantly hampered. If drying occurs below the T_g' , water will be rapidly removed without disrupting the microstructure established upon freezing which minimizes product loss during lyophilization [F. Franks, *Develop. Biol. Stand.*, 74:9-19 (1991)]. Thus, the T_g' of a preparation sets the safe upper temperature limit for primary drying. Because T_g' can vary over an enormous range (-1 to -50°C), determination of this value for a proposed formulation is extremely important and the first step in process development.

The T_g' of a preparation is very concentration dependent: buffer salts, amino acids, carbohydrates and other components added to a formulation can influence T_g' [te Booy, M.P.W.M., et al, *Pharmaceutical Res.*, 9(1):109-114 (1992)]. Differential scanning calorimetry (DSC) gives a direct measurement of T_g' [Her, L.M., et al., *Pharmaceutical Research*, 11(1): 54-59 (1994); Hatley, R.H.M., *Developments in Biological Standardization*, 74: 105-122 (1990); Hatley, R.H.M.a.F., F., *Journal of Thermal Analysis*, 37: 1905-1914 (1991)].

DSC analysis was performed with a MDSC 2920 (TA Instruments, New Castle, DE) equipped with liquid nitrogen cooling. The calorimeter was calibrated for temperature and cell constant using indium. Fifty microliters of each formulation were analyzed in hermetically sealed aluminum pans (TA Instruments). The cooling rate was 1 °C/min. The temperature range was -60 to +25 °C. Glass transition values (T_g') are reported as the midpoint of the observed transition. Experiments were performed in duplicate.

Sucrose (1M) in potassium phosphate buffered saline has a glass transition of -34.5 °C (see Table 8 below).

Table 8

	Formulation	Particles/ml	Tg' (°C)
	1 M Sucrose	0	-34.5
	1 M Sucrose	1×10^{11} Ad	-33.9
5	1 M Sucrose	1×10^{12} Ad	-32.0
	0.5 M Sucrose	0	-33.5
	0.5 M Sucrose	1.55×10^{11} AAV	-34.71
	0.5 M Sucrose	1.53×10^{12} AAV	-35.37

When adenovirus was added at a concentration of 1×10^{11} particles/ml, the Tg' rose to -33.9°C . Addition of another log of virus pushed the glass transition temperature to -32.0°C . This effect is commonly seen with proteins [M. J. Pikal, *Biopharm*, September:18-27 (1990); M.J. Pikal, *Pharmaceutical Res.*, 8(4):427-437 (1991)]. As protein replaces the water in a preparation, the glass transition rises. Adeno-associated virus had a different effect on the Tg' of a 0.5M sucrose formulation. Addition of the virus at a concentration of 1.55×10^{11} genome copies/ml lowered the glass transition temperature from -33.5 to -34.71°C . Addition of another log of virus dropped Tg' to -35.37°C . These results suggest that this virus may interact with excipients in the frozen state differently than traditional proteins. Based on this information, the primary drying temperature for the Ad preparation was set at -35°C . AAV was dried at -38°C .

Example 21 - Effect of Final Moisture Content of the Lyophilized Cake on the Recovery of Adenovirus

Various blends of sucrose and mannitol were tested for final moisture content after a standard lyophilization cycle (-40°C for 2 hours, -35°C for 11 hours, -10°C for 2 hours, 0°C for 2 hours and 25°C for 3 hours at a pressure of 30 mtorr).

Water content of lyophilized formulations was determined by Karl Fischer titration [J.C. May, et al., *Dev. Biol. Stand.*, 74:153-164 (1992)] and confirmed by thermogravimetric analysis (TGA) [J.C. May, et al., *J. Biol. Standardization*, 10:249-259 (1982)]. For Karl Fisher titration, an Aqua Star Karl Fischer titrator (Model C3000, EM Sciences) equipped with a fritless cell was calibrated with Karl Fischer water standard (2-methoxy ethanol) and blanked with the addition of 1ml anhydrous methanol (EM Sciences). Lyophilized samples were reconstituted with anhydrous methanol and added to the titrator. TGA was performed with a Perkin-Elmer TGA 7 Series thermogravimetric system. Three to five milligrams of lyophilized preparation was scanned at 10°C/min from 25 to 200°C.

The data provided in Table 9 below are the average of 10 vials from a single lyophilization run.

Table 9. Final Moisture Content of the Lyophilized Cake Significantly Affects Recovery of Adenovirus.

15	Sucrose:Mannitol	Pre-Lyo	Post-Lyo	Moisture Content
	Ratio	Titer (ifu/ml)	Titer (ifu/ml)	
	1:4	$5.12 \pm 0.01 \times 10^{11}$	$4.6 \pm 0.2 \times 10^{10}$	$0.6 \pm 0.01\%$
	1:2	$5.12 \pm 0.01 \times 10^{11}$	$2.8 \pm 0.11 \times 10^{11}$	$0.9 \pm 0.001\%$
	3:4	$5.12 \pm 0.01 \times 10^{11}$	$4.77 \pm 0.04 \times 10^{11}$	$1.3 \pm 0.07\%$
20	1:1	$5.12 \pm 0.01 \times 10^{11}$	$5.08 \pm 0.1 \times 10^{11}$	$1.4 \pm 0.25\%$
	2:1	$5.12 \pm 0.01 \times 10^{11}$	$2.89 \pm 0.3 \times 10^{11}$	$1.6 \pm 0.1\%$
	4:1	$5.12 \pm 0.01 \times 10^{11}$	$1.0 \pm 0.22 \times 10^{11}$	$3.35 \pm 0.05\%$

When adenovirus was added to these preparations at a concentration of 5.12×10^{11} ifu/ml, recovery of infectious virus was extremely sensitive to the final moisture content of the product (Table 9). A preparation of 1:4 sucrose:mannitol had a final moisture content of 0.6% and suffered a 1.1 log loss in viral titer. The 3:4 ratio, with a moisture content of 1.3%, lost less than 1 log of infectious virus. A preparation consisting of a 1:1 ratio of sucrose:mannitol did not experience any loss

of titer during the entire lyophilization process. The moisture content of this preparation was 1.4%. If water content rose 0.2% beyond this to 1.6%, titer fell by 2.2 log units. These data indicate that adenovirus is not only sensitive to the final water content in the lyophilized product, but that there is a rather narrow window in which viral titer can be maintained. As a result, all adenoviral lyophilization processes were tailored to produce a final moisture content of 1.3-1.5% in order to afford maximum recovery of virus.

Example 22 - Effect of Virus Concentration on Recovery of Viral Titer after Lyophilization

In order to assess the maximum allowable concentration of adenovirus that facilitates optimal recovery of viral titer after lyophilization, adenovirus was lyophilized at two different concentrations in two separate formulations. The results provided in Table 10 are the averages of 10 vials of each preparation.

Table 10. Effect of Initial Adenoviral Concentration on the Recovery of Infectious Virus after Lyophilization.

Formulation	Pre-Lyo Titer (lfu/ml)	Post-Lyo Titer (lfu/ml)
Sucrose:Mannitol		
Ratio		
1:4	$5.2 \pm 0.04 \times 10^{10}$	$4.6 \pm 0.01 \times 10^9$
1:4	$5.5 \pm 0.1 \times 10^{11}$	$2.97 \pm 0.1 \times 10^{11}$
4:1	$1.9 \pm 0.2 \times 10^{10}$	$1.0 \pm 0.02 \times 10^9$
4:1	$2.0 \pm 0.14 \times 10^{11}$	$1.6 \pm 0.1 \times 10^{11}$

In general, preparations with higher initial titers produced better recovery of active virus after lyophilization. Preparations at low viral concentration (2.5×10^{10} lfu/ml) suffered a loss of approximately one log of virus regardless of formulation. The loss in titer was less than one log at higher adenovirus concentrations (2.5×10^{11} lfu/ml). This is believed to be in part due to the ability of the virus itself to assist in maintaining the final frozen pH of a preparation upon freezing at high

concentrations (data not shown). Adenovirus preparations of $1-5 \times 10^{11}$ lfu/ml were used in additional lyophilization studies.

Example 23 - Stability of Lyophilized Viral Vectors

After careful evaluation of process and formulation effects, a large-scale
5 preparation of adenovirus (1×10^{11} lfu/ml) was lyophilized in a 1M sucrose formulation under the following protocol: -40°C for 2 hours, -35°C for 9 hours, -10°C for 4 hours, 0°C for 4 hours and 25°C for 8 hours at a pressure of 30 mtorr. After lyophilization, some vials were reconstituted.

The only loss detected in this preparation after one year at 4°C was the
10 initial loss of 0.5 log of virus due to the process itself. Virus from the same lot prepared in 10% glycerol and stored at -20°C suffered a drop in titer of approximately 2 logs over the same time period. The stability of reconstituted preparations stored at 4°C was also assessed. Virus titer dropped at a steady rate of 0.5 log/day over a period of 4 days, indicating that the vector must be used soon
15 after reconstitution. This degradation rate was similar for other lyophilized preparations regardless of formulation (data not shown). Studies to elucidate the nature of this rapid decline in titer and develop novel admixtures for reconstitution which promote viral stability are currently underway.

Little work was done with respect to the development of formulations and
20 optimization of the lyophilization process for AAV vectors. In a single experiment, half of an AAV preparation (8.7×10^8 lfu/ml) was desalted into potassium phosphate buffered saline. The remaining half was desalted in a formulation of 0.4% sucrose, 0.4% mannitol and protamine. Each preparation was lyophilized under the following protocol: -40°C for 2 hours, -38°C for 11 hours, -10°C for 2 hours, 0°C
25 for 2 hours, and 25°C for 3 hours at a pressure of 30 mtorr.

After an initial loss of 0.3 log in titer due to the lyophilization process, the formulated preparation did not experience any loss in infectivity for 90 days at 25°C . It is also important to note that the preparation prepared in buffer without any

additional excipients (Buffer) experienced a similar loss in titer after lyophilization. This suggests that AAV may be resistant to the stresses of the freeze-drying process.

5 All publications cited in this specification are incorporated herein by reference herein. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for conjugating a recombinant virus with polyethylene glycol to enhance transduction efficiency thereof, said method comprising the steps of:
 - a) reacting activated polyethylene glycol and a recombinant virus at ambient temperature for about 1 to about 2 hours; and
 - b) stopping the reaction, thereby obtaining polyethylene glycol conjugated virus.
2. The method according to claim 1, wherein the activated polyethylene glycol and the recombinant virus are reacted at a ratio of about 10: 1 polyethylene glycol to virus.
3. The method according to claim 1 or claim 2, wherein the reaction takes place in solution and the recombinant virus is present at a concentration of about 1×10^{10} to about 1×10^{15} particles per ml of solution.
4. The method according to any of claims 1 to 3, wherein the polyethylene glycol is activated with a compound selected from the group consisting of tresyl chloride, succinimidyl succinate and cyanuric chloride.
5. The method according to any of claims 1 to 4, wherein the polyethylene glycol has a molecular weight 5000.
6. The method according to any of claims 1 to 5, wherein the polyethylene glycol is a monomethoxypolyethylene glycol.
7. The method according to any of claims 1 to 6, wherein said recombinant virus is adeno-associated virus.

8. The method according to any of claims 1 to 6, wherein said recombinant virus is an adenovirus.
9. A polyethylene-glycol conjugated virus, said virus prepared according to the method of any of claims 1 to 7.
10. The virus according to claim 9, wherein said virus is selected from the group consisting of an adenovirus and an adeno-associated virus.
11. The virus according to claim 9, wherein said virus is conjugated with monomethoxypolyethylene glycol.
12. A method for increasing transduction efficiency of a recombinant virus, method comprising the steps of:
 - delivering a modified recombinant virus according to claim 9 to host cells.
13. A method for re-administration of a molecule to a selected host cell via a viral vector, said method comprising the steps of:
 - (a) contacting the host cell with a polyethyleneglycol (PEG)-modified virus according to claim 9, wherein said virus comprises a molecule for delivery to a host cell; and
 - (b) contacting the host cells with a recombinant virus comprising the molecule.
14. The method according to claim 13, wherein the host cells are contacted with the PEG-modified virus subsequent to contacting the host cells with a recombinant virus comprising the molecule.

15. The method according to claim 13, wherein the recombinant virus comprising the molecule is a second PEG-modified virus.

16. The method according to claim 15, wherein in the first PEG-modified virus and the second PEG-modified virus, the PEG is monomethoxy PEG (MPEG) and for each virus, the MPEG has been activated by different groups.

17. The method according to claim 16, wherein the MPEG is activated with a compound selected from the group consisting of tresyl chloride, succinimidyl succinate and cyanuric chloride.

18. A composition useful for delivery of a selected molecule to host cells, said composition comprising a polyethylene-glycol conjugated virus prepared according to the method of any of claims 1 to 7 and a physiologically acceptable carrier.

19. A composition that enhances the physical stability of viral vectors, said composition comprising:

- (a) a recombinant viral vector comprising a molecule for delivery to host cells;
- (b) sucrose; and
- (c) mannitol, wherein the ratio of sucrose to mannitol is about 1 to about 1.

20. The composition according to claim 19, wherein the composition is lyophilized to a final moisture content of about 1.2% to about 1.7%.

21. The composition according to claim 19, wherein the composition in solution comprises about 1×10^{10} to about 1×10^{15} particles recombinant virus per milliliter solution.

22. The composition according to claim 19, further comprising a beta cyclodextrin.

23. The composition according to claim 19, further comprising a protamine.

1/2

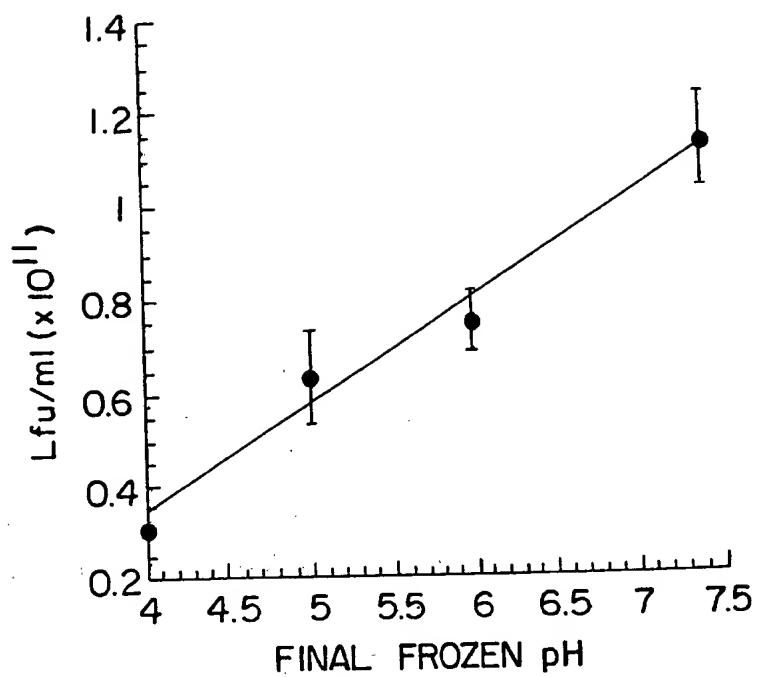


FIG. 1A

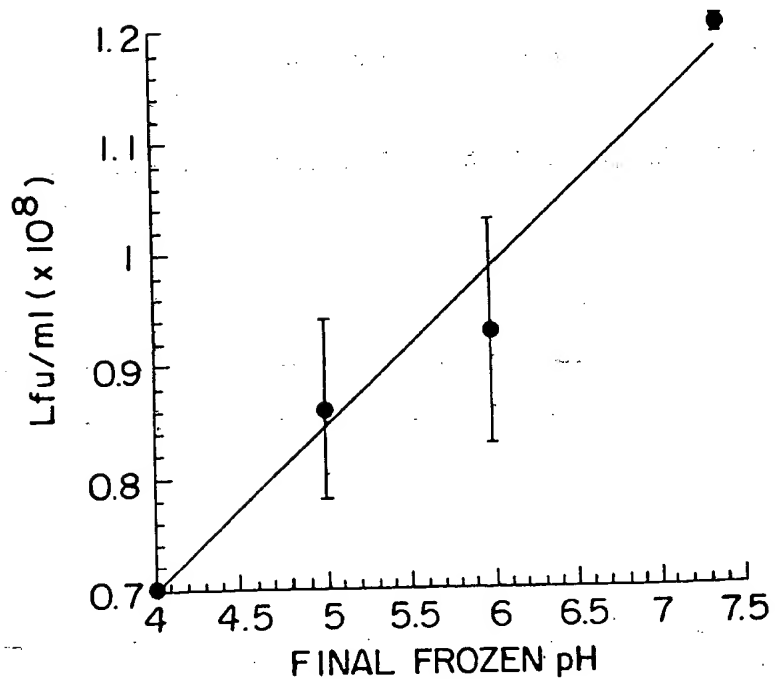


FIG. 1B

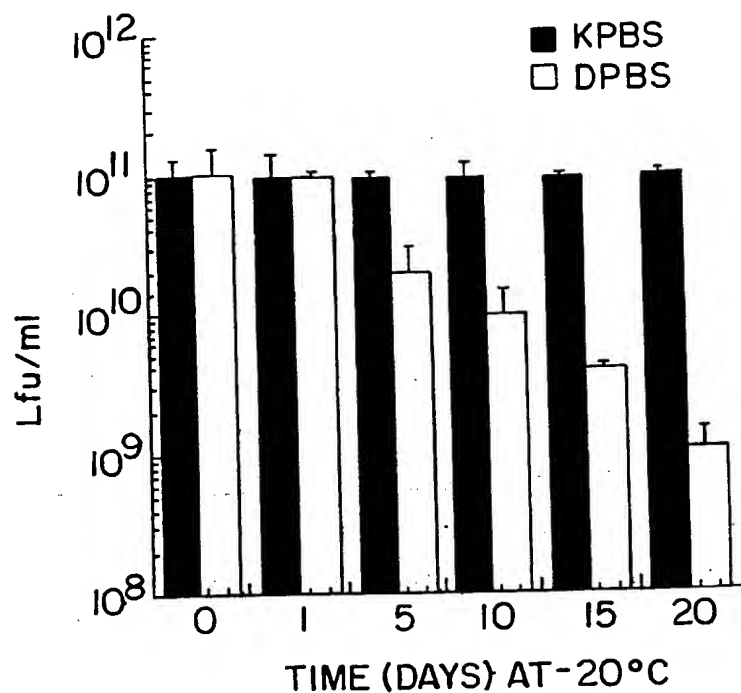


FIG. 1C

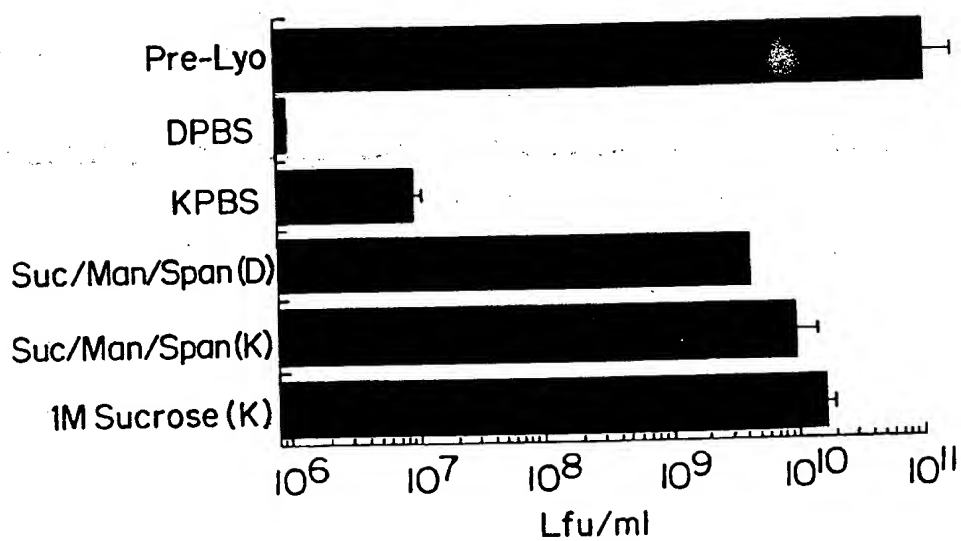


FIG. 2

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(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

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14 March 2002

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WO 01/23001 A3

(54) Title: RAPID PEG-MODIFICATION

(57) **Abstract:** A rapid method for modifying a viral capsid or envelope protein with a polyethylene glycol (PEG) is described. Also provided are methods of delivering a molecule using the PEG-modified viruses of the invention. Compositions containing the PEG-modified viruses of the invention, are characterized by improved gene expression, reduced neutralizing antibody and CTL production. Also provided are viral compositions having enhanced physical stability, in which the viruses are lyophilized in a formulation having a 1:1 ratio of sucrose and mannitol are provided.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/26449

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 98 44143 A (DELGADO CHRISTINA ; FRANCIS GILLIAN E (GB); POLYMASC PHARMACEUTICAL) 8 October 1998 (1998-10-08) abstract; claims 8,10-12,25,40; examples 1-3	1-18
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	O'RIORDAN C. R. ET AL: "PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo" HUMAN GENE THERAPY, vol. 10, 20 May 1999 (1999-05-20), pages 1349-1358, XP000857510 abstract see discussion page 1350, column 2 ---	1-18
X,P	CROYLE M. A. ET AL: "Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions" HUMAN GENE THERAPY, vol. 11, 10 August 2000 (2000-08-10), pages 1713-1722, XP001023031 abstract see discussion page 1714, column 2, paragraph 1 ---	1-23
P,A	OHSAWA T. ET AL: "Enhancement of adenovirus-mediated gene transfer into dermal fibroblasts in vitro and in vivo by polyethylene glycol 6000" JOURNAL OF DERMATOLOGY, vol. 27, April 2000 (2000-04), pages 244-251, XP001020884 see discussion abstract ---	1-18
T	CROYLE M A ET AL: "STEALTH ADENOVIRUSES BLUNT CELL-MEDIATED AND HUMORAL IMMUNE RESPONSES AGAINST THE VIRUS AND ALLOW FOR SIGNIFICANT GENE EXPRESSION UPON READMINISTRATION IN THE LUNG" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 75, no. 10, May 2001 (2001-05), pages 4792-4801, XP001015886 ISSN: 0022-538X abstract; figure 1 --- -/--	1-23

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/26449

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CROYLE M A ET AL: "DEVELOPMENT OF NOVEL FORMULATIONS THAT ENHANCE ADENOVIRAL-MEDIATED GENE EXPRESSION IN THE LUNG IN VITRO AND IN VIVO" MOLECULAR THERAPY, ACADEMIC PRESS, SAN DIEGO, CA,, US, vol. 4, no. 1, July 2001 (2001-07), pages 22-28, XP001023435 ISSN: 1525-0016 see discussion abstract	19-23
X	FISHER, DEREK ET AL: "Factors with impact on the success of protein and virus PEGylation" POLYM. PREPR. (AM. CHEM. SOC., DIV. POLYM. CHEM.) (2000), 41(1), 1012-1013 , XP001051338 see experimental	1-23
X	CROYLE MARIA A ET AL: "Beta cyclodextrins enhance adenoviral- mediated gene delivery to the intestine" PHARMACEUTICAL RESEARCH, NEW YORK, NY, US, vol. 15, no. 9, 1998, pages 1348-1355, XP000953222 ISSN: 0724-8741 abstract	19-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/26449

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-23
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11,18

Method of conjugating a recombinant virus with polyethylene glycol.

2. Claim : 12

Use of a conjugate of recombinant virus with polyethylene glycol for increasing transduction efficiency of a recombinant virus.

3. Claims: 13-17

Use of a conjugate of recombinant virus with polyethylene glycol for readministration of a molecule via a viral vector comprising: a) contacting the host cell with a polyethylene glycol modified virus comprising the molecule, b) contacting the host cell with recombinant virus comprising the molecule

4. Claims: 19-23

A composition that enhances the physical stability of viral vectors comprising: a) a recombinant viral vector comprising a molecule for delivery to host cells, b) sucrose, c) mannitol

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-23

Present claims relate to a compound/method defined (inter alia) by reference to the following parameters: "a recombinant virus", "activated polyethylene glycol". The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is not fully possible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search for the first invention has been restricted to the parts relating to the compounds/methods mentioned in the examples and the activated PEG and recombinant virus specifically mentioned in claims 4-8,10,11,16,17,19-23

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/26449

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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			AU 6445698 A	22-09-1998
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			EP 0994959 A1	26-04-2000
			WO 9844143 A1	08-10-1998

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